UNIVERSITY OF NAPLES
"FEDERICO II"

DOCTORATE PROGRAM IN
CLINICAL AND EXPERIMENTAL MEDICINE

Metabolic Endocrine Curriculum

XXIX CYCLE (2014-2017)

Coordinator: Prof. Gianni Marone

PHD THESIS

Understanding Type 2 Diabetes: Epigenetic profiling in populations at high risk for the disease

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Understanding Type 2 Diabetes: Epigenetic profiling in populations at high risk for the disease

Table of contents

List of Publications

Parrillo L, Costa V, Raciti GA, Longo M, Spinelli R, Esposito R, Nigro C, Vastolo V, Desiderio A, Zatterale F, Ciccodicola A, Formisano P, Miele C, Beguinot F. *Hoxa5* undergoes dynamic DNA methylation and transcriptional repression in the adipose tissue of mice exposed to high-fat diet. Int J Obes (Lond). 2016 Jun;40(6):929-37.

Desiderio A, Spinelli R, Ciccarelli M, Nigro C, Miele C, Beguinot F, Raciti GA. Epigenetics: spotlight on type 2 diabetes and obesity. J Endocrinol Invest. 2016 Oct;39(10):1095-103.

Raciti GA, Spinelli R, Desiderio A, Longo M, Parrillo L, Nigro C, D'Esposito V, Mirra P, Fiory F, Pilone V, Forestieri P, Formisano P, Pastan I, Miele C, Beguinot F. Specific CpG hyper-methylation leads to *Ankrd26* gene down-regulation in white adipose tissue of a mouse model of diet-induced obesity. Sci Rep. 2017 Mar 7;7:43526.

Abstract

The epigenetic hypothesis argues that, in addition to genetic variation, epigenetics provides an additional set of mechanisms mediating the relationship between genotype and the environment that may contribute to the individual susceptibility to different disorders such as T2D.

The aim of this study was to investigate the epigenetic profiling in two population at high risk of T2D, i) obese and ii) first degree relatives of type 2 diabetic patients (T2D-FRDs), using a candidate gene study and an EWAS approach, respectively.

i) In human Peripheral Blood Leucocytes (PBLs), the *ANKRD26* gene expression inversely correlates with BMI (r=-0.436; $p<0.01$), and was lowered by about 25% in obese subjects compared with lean individuals ($p<0.01$). Additionally, in the same individuals a site specific CpG hyper-methylation of the *ANKRD26* promoter occurred in obese compared with lean subjects ($p<0.001$) and inversely correlates with the *ANKRD26* gene expression (r=-0.850, $p<0.05$).

ii) In Subcutaneous Adipose Tissue Stromal-Vascular Fraction cells (SVFs) from lean euglycaemic T2D-FDRs, 34 miRNAs and 84 genes were identified to be differentially expressed compared with the lean counterpart with no family history of T2D (control group) by Next Generation Sequencing. In addition, in the T2D-FDRs, the expression of the miRNAs, *hsa-miR-23a-5p*, *-193a-5p* and *-193b-5p*, was down-regulated compared with the control subjects ($p<0.01$), and inversely correlates with adipocyte cell size ($p<0.01$). Interestingly, bio-informatic analysis of these data highlighted that the expression changes of the miRNAs, *hsa-miR-23a-5p*, *-193a-5p* and *-193b-5p*, enriched pathways associated to adipocyte commitment/differentiation and function. Furthermore, the expression of the miRNAs target gene *IGF2* and *MXRA5* was up-regulated in the T2D-FDRs compared with controls ($p<0.01$), and inversely correlates with miRNA expression ($p<0.01$) and positively with adipocytes cell size ($p<0.01$).

In conclusion, in the first study I evidenced that the down-regulation of the *ANKRD26* gene and the site specific CpG hyper-methylation of its promoter represent a common abnormality in obese patients. In the second study, I instead concluded that specific changes in the expression of the miRNAs, *hsa-miR-23a-5p*, *-193a-5p* and *-193b-5p*, occurred in the individuals with familiarity for T2D and may cause adipose dysfunction and impaired adipose cell recruitment, which are typical in T2D-FDRs, by interfering with functions of adipocyte specific pathways.

Background

1.1 Diabetes mellitus

Diabetes mellitus (DM) is a group of metabolic diseases characterized by chronic hyperglycemia [1]. There are two main types of DM: i. Type 1 diabetes (T1D), once called insulin dependent diabetes mellitus (IDDM), caused by lack of insulin secretion by beta cells of the pancreas [1], and ii. Type 2 Diabetes (T2D), once called non-insulin dependent diabetes mellitus (NIDDM), mainly caused by decreased sensitivity of target tissues to insulin [1]. The classification of DM is reported in Table 1.

Type 1 Diabetes	**A. Autoimmune** **B. Idiopathic**	
Type 2 Diabetes	1. Insulin resistance predominates over the relative defects in hormone secretion 2. Defects in insulin secretion predominate over the presence of insulin resistance	
Other types of diabetes	**A. Gestational diabetes** **B. Genetic defects in β-cell function:** 1. Chromosome 12, HNF-1α (MODY 3) 2. Chromosome 7, glycosidase (MODY 2) 3. Chromosome 20, HNF-4α (MODY 1) 4. Mitochondrial DNA 5. Others **C. Genetic defects in insulin action** 1. Type A insulin resistance 2. Leprechaunism 3. Rabson-Mendenhall syndrome 4. Lipotrophic diabetes 5. Others **D. Disease of the exocrine pancreas** 1. Pancreatitis 2. Pancreatectomy/trauma 3. Neoplasia 4. Cystic fibrosis 5. Hemochromatosis 6. Fibrocalcific pancreatopathy 7. Others **E. Endocrinopathies** 1. Acromegaly 2. Cushing syndrome 3. Glucagonoma 4. Pheochromocytoma 5. Hyperthyroidism 6. Somatostatinoma 7. Aldosteronoma 8. Other	**F. Pharmacologically or chemically induced** 1. Vacor 2. Pentamidine 3. Nicotinic acid 4. Glucocorticoids 5. Thyroid hormones 6. Diazoxide 7. β-adrenergic agonists 8. Tiazides 9. Dilantin 10. α interferon 11. Others **G. Infections** 1. Congenital rubeola 2. Cytomegalovirus 3. Others **H. Infrequent forms of autoimmune** diabetes 1. Stiff-man syndrome) 2. Antibodies against insulin receptors 3. Others **I. Other syndromes occasionally** associated with diabetes 1. Down syndrome 2. Klinefelter syndrome 3. Turner syndrome 4. Wolfram syndrome 5. Friedreich ataxia 6. Huntington's chorea 7. Lawrence-Moon-Biedel syndrome 8. Myotonic dystrophy 9. Porphyria 10. Prader-Willi syndrome 11. Others

Table 1. Classification of diabetes. MODY: mature onset diabetes of the young. Modified from Diabetes Care. 2009;32(Suppl 1):S62-S67.

Since the origin and etiology may be different, the DM types share the existence of defects in insulin secretion, insulin action, or both. The reduced sensitivity to insulin is often called insulin resistance. The basic effect of insulin lack or insulin resistance on glucose metabolism is an impairment of the efficient uptake and utilization of glucose by most cells of the body, except those of the brain [2]. As a result of this, blood glucose concentration increases, cell utilization of glucose falls and utilization of fats and proteins increases [3]. The classic symptoms in T1D, are excessive secretion of urine (polyuria), thirst (polydipsia), weight loss and tiredness; these symptoms may be less marked in T2D [1]. Indeed, in this form, no early symptoms appear and the disease is diagnosed several years after its onset, when complications are already present. T2D represents the more frequently occurring form, which accounts for ~90% of all diabetes cases worldwide [4]. It occurs commonly in adults, but is being noted increasingly in adolescents as well. The International Diabetes Federation revealed that about 382 million people worldwide, or 8.3% of adults, are estimated to have diabetes. If these trends continue, by 2035, some 592 million people, or one adult in 10, will have diabetes [5]. These numbers make it clear the high interest about the pathogenesis of T2D, both in the scientific research and in the field of clinical trials.

1.2 Type 2 Diabetes

T2D is a complex multifactorial disease whose incidence has increased in both developed and developing countries worldwide. The combined effect of population aging, urbanization, dietary changes, physical inactivity and unhealthy behaviors, are driving the emerging pandemic of T2D [6]. The pathology is characterized by sustained elevations of plasma glucose levels resulting in chronic hyperglycemia. The onset of chronic hyperglycemia is associated with long-term damage and dysfunction and typically results when insulin secretion from the islets fails to keep pace with increasing insensitivity to the action of circulating insulin on its target tissues [1]. Insulin resistance is an early feature of T2D. The major sites of insulin resistance include liver and the peripheral tissues, skeletal muscle and fat. In the Figure 1 is represented the cross-talk between tissues in the regulation of glucose metabolism. In muscle and fat, insulin resistance is manifested by decreased glucose uptake. In particular, in muscle, insulin resistance impaired the utilization of glucose by non-oxidative pathways and by the decrease in glucose oxidation as well. In the fat, insulin resistance increase free fatty acid (FFA) flux from adipose tissue to non-adipose tissue, resulting in abnormalities of fat metabolism, while in the liver insulin resistance leads to failure of insulin to suppress hepatic glucose production, which is fueled by glycogen breakdown and particularly by gluconeogenesis [7]. T2D often remains asymptomatic and undetected for years. The failed diagnosis is connected to serious complications, which can result in early death. Long-term complications of diabetes include retinopathy with potential loss of vision; nephropathy leading to renal failure; peripheral neuropathy; autonomic neuropathy and cardiovascular disease.

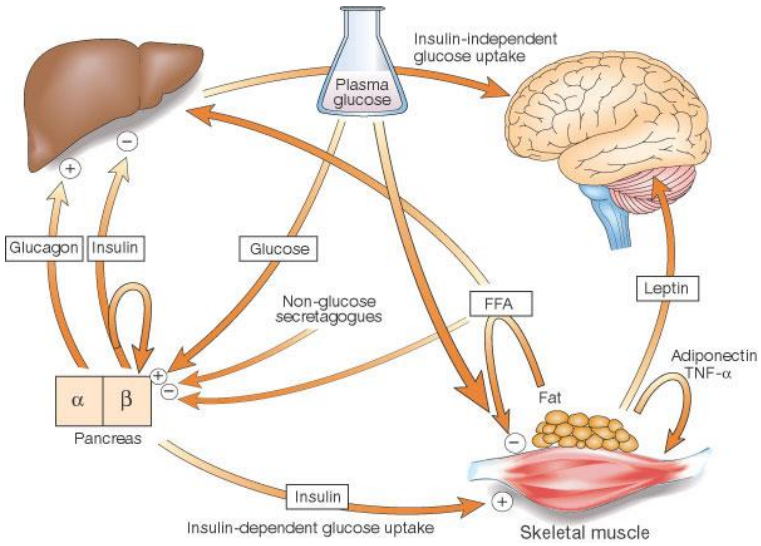

Figure 1. Cross-talk between tissues in the regulation of glucose metabolism. Nature. 2001 Dec 13;414(6865):799-806.

Nevertheless, diabetic-patients have an increased incidence of atherosclerotic cardiovascular, peripheral arterial, and cerebrovascular diseases, besides hypertension and abnormalities of lipoprotein metabolism as well [1]. Although the pathogenesis of diabetes is complex, several risk factors have been identified. Among these, obesity, family history of diabetes, aging, physical inactivity, impaired glucose tolerance, history of gestational diabetes are the most relevant [6]. In Table 2 are reported the odds ratio score for T2D risk.

Variable	Diabetes (95%CI) odds
Age ≥45 years	3.3 (2.4-4.6)
Female gender	1.6 (1.2-2.2)
Family history of diabetes	1.9 (1.3-2.0)
BMI 25-30	1.6 (1.5-1.8)
30-35	3.2 (2.9-3.5)
35-40	5.9 (5.3-6.5)
>40	11.6 (10.5-12.8)
Central obesity	2.4 (1.8-3.3)
Physical inactivity	1.4 (1.1-1.9)

Table 2. Odds ratio score for diabetes risk. BMI: Body mass index, **CI:** Confidence interval. Modified from J Family Med Prim Care. 2015 Jul-Sep;4(3):399-404, Diabetol Metab Syndr. 2014 Apr 3;6(1):50.

1.3 Risk factors of T2D

1.3.1 Obesity

The increasing global prevalence of T2D is tied to rising rates of obesity in part as consequence of social trends toward alterations of the energy homeostasis in terms of balance among energy intake, expenditure and storage [8]. Obesity is one of the major risk factors for T2D and it has shown a parallel increase in global prevalence. Indeed, the estimated 205 million men and 297 million women worldwide (as of 2008) to be obese (body mass Index, BMI ≥30-35 kg/m^2) are at 10-40 fold higher risk of T2D [9]. The relatively recent rise in obesity appears to be related to gene-environment interactions where the genetic background, coupled with the current obesogenic environment, and the rewarding nature of palatable foods, tends to promote obesity [10]. Nevertheless, some obese people never develops T2D, and some T2 diabetics are extremely lean. It is now recognized that among obese people 1 on 3 are "metabolically healthy", which means that their fasting glucose, triglycerides, and other metabolic markers are normal. This population is at no higher risk for T2D or cardiovascular disease (CVD) than their metabolically healthy lean counterparts [11]. How this might be possible is still unclear. Recent growing evidences have raised much interest in the potential role of regional adipose tissue (AT) distribution in the contribution to T2D. AT is an endocrine organ involved in a number of processes including the communication between the brain and peripheral tissues by secreting hormones which regulate appetite and metabolism [12]. These functions appear to be modulated by the location of the visceral AT (VAT) and subcutaneous AT (scAT), by the size of the average adipocyte in the tissue, and by adipocyte metabolism of glucose and corticosteroids. Thus, fat distribution plays an important role in metabolic risk. Indeed, increase in intra-abdominal visceral fat promotes a high risk of metabolic disease, whereas increased subcutaneous fat in the thighs and hips exerts little or no risk [13]. Nevertheless, it is now known that abdominal subcutaneous fat cell size in humans is negatively correlated with insulin sensitivity independently to degree of obesity [14] and abdominal subcutaneous adipose cell size has been shown to be an independent predictor of future T2D in perspective studies [15].

1.3.2 Family history of T2D

A family history of diabetes is associated with a range of metabolic abnormalities and is a strong risk factor for the development of T2D [16]. Familiarity for T2D has long been adopted as an argument supporting the genetic origin of diabetes, indeed, first degree relatives of T2D patients (T2D-FDRs) have up to 10-fold higher risk of developing the disease than age- and weight-matched subjects without a clear family history of the disease [17, 18]. In support of this, the 39 % of T2 diabetic individuals have at least one affected parent and among the monozygotic twin pairs with one affected twin, approximately the 70 % of unaffected twins eventually develop the disease

[18]. It is likely that this elevated risk is mediated, in part, by both genetic and shared environmental components among family members, but the precise factors accounting for this increased risk are poorly understood. Different studies suggest that T2D risk depends not only on the number of affected relatives but on the age at diagnosis and on whether maternal or paternal history is present as well [19]. Particularly, maternal diagnosis provides further insight into individual risk of T2D. Indeed, it has been reported that individuals with a young maternal age at diagnosis of T2D had a greatly increased odds of impaired glucose tolerance differently for individual with younger paternal diagnosis [20]. *Scott et al.* have also observed in their study that the family history associated-risk is 17% for maternal diabetes and less than 1% for paternal diabetes [20]. This evidence suggests that the risk of T2D attributable to family history may have a distinct etiology depending on the family member affected. In addition, T2D-FDRs frequently have an impaired nonoxidative glucose metabolism (indicative of insulin resistance) long before the onset of T2D and may have also beta-cell dysfunction, as evidenced by decreases in insulin and amylin release in response to glucose stimulation [18]. Furthermore, recent evidence suggests that family history of T2D is accompanied by scAT dysfunction, as well [21].

1.3.3 scAT dysfunction: the connecting bridge among obesity and familiarity of T2D

It is now well recognized that a limited expandability of the scAT leads to inappropriate adipose cell expansion (adipocyte hypertrophy) with local inflammation and dysregulated insulin-resistant AT [22] (Figure 2). Furthermore, obesity with abdominal subcutaneous adipose cell enlargement is also characterized by a reduced number of pre-adipocytes that can undergo differentiation [23]. This is due to specific inability of the pre-adipocytes in abdominal scAT to differentiate into mature adipocytes and/or of the progenitor cells to be committed into adipocyte lineage. This intriguing finding explains in part why there is differential lipid partitioning and why abdominal obesity is closely linked to insulin resistance. Thus, the inability to store excess fat in the scAT is likely a key mechanism for promoting ectopic fat accumulation in tissues and areas where fat can be stored, including the intra-abdominal and visceral areas, the liver, epi/pericardial area, around vessels, the myocardium, and skeletal muscle tissue [24]. It was also demonstrated that pre-adipocytes of T2D subjects display an intrinsic gene expression profile of decreased differentiation capacity. Indeed, the decreased expression of genes involved in differentiation can provide a molecular basis for the reduced adipogenesis of pre-adipocytes from T2D subjects, leading to reduced formation of adipocytes in subcutaneous fat depots and ultimately to ectopic fat storage in these subjects [25]. Recent evidence revealed that even non-obese T2D-FDRs are less insulin-sensitive and have considerably larger subcutaneous fat cells than individuals with the same age, BMI, amount of body fat but lacking a known family history of diabetes. This finding indicates

that T2D-FDRs, also when non-obese, have a dysfunctional scAT characterized by inappropriate hypertrophy of the adipose cells. Overall the combinations of these observations suggest that familiarity for T2D, as well as obesity and T2D itself, presents in the scAT a common feature due to an impaired ability to recruit and/or differentiate new adipose cells [23, 25-26]. The reasons for this inability are currently unclear and deserve further investigations.

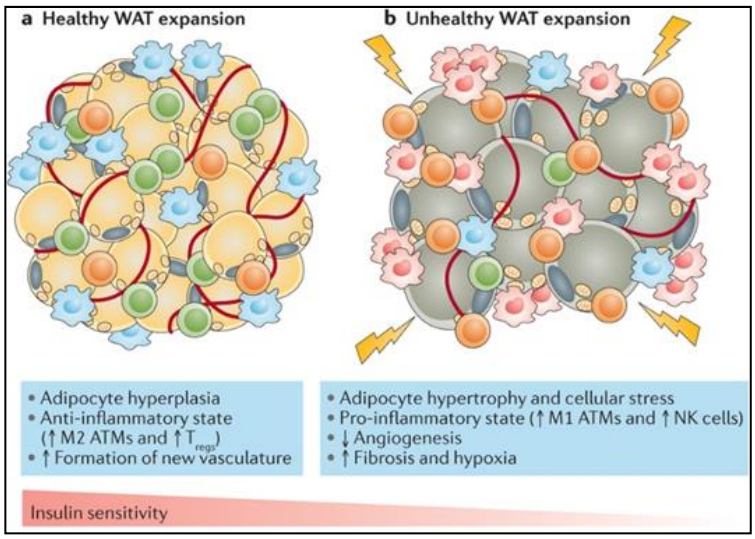

Figure 2: Metabolically healthy white adipose tissue expansion versus unhealthy pathological expansion. a) Healthy WAT expansion displays high insulin sensitivity and is characterized by an anti-inflammatory state due to elevated levels of Treg cells and M2 adipose tissue macrophages (ATMs), sufficient vasculature to support tissue expansion and adipocyte hyperplasia. **b)** Unhealthy WAT expansion, instead, displays a heightened state of inflammation (high levels of infiltrating pro-inflammatory M1 ATMs and NK cells), impaired formation of new vasculature to support WAT growth, and enhanced fibrosis and hypoxia. Such events contribute to the development of WAT insulin resistance. Nat Rev Drug Discov. 2016 Sep;15(9):639-60.

1.3.4 The genetic basis of T2D and obesity

Until 2012, more than 175 loci have been associated with common forms of T2D and closely related continuous traits such as fasting glucose, BMI, and fat distribution [27-29]. Population and family (including twins) studies have extensively documented the familial aggregation of both T2D and obesity [18]. In particular for T2D risk, linkage studies in parallel with candidate gene studies have reported many T2D-associated loci (Figure 2) [30]. Among them, the strongest known T2D-associated locus, with an odds ratio for T2D of 1.4-fold per allele, was mapped at the *TCF7L2* (*transcription factor 7-like 2*) locus [31]. This gene was attested to modulate the pancreatic islet function. Indeed, most studies suggest that the predominant intermediate phenotype associated with *TCF7L2* variation is the impaired insulin secretion [31].This is consistent with the replicated observation that the *TCF7L2* association is greater among

lean than obese T2D subjects [31]. The first genome-wide association study (GWAS) for T2D provided the evidence that GWAS would work for complex disease and beside *TCF7L2,* single nucleotide polymorphisms (SNPs) in the zinc transporter *SLC30A8* and variants in *HHEX* were identified, as well [32]. This study was successively followed by other that found novel associations at *CDKAL1*, *IGF2BP2* and *CDKN2A/B* genes [32]. The Diabetes and Genetics Replication and Meta-Analysis (DIAGRAM) consortium identified other six new loci *JAZF1*, *CDC123-CAMK1D*, *TSPAN8-LGR5*, *THADA*, *ADAMTS9* and *NOTCH2* [30]. Subsequently, the Meta-Analyses of Glucose and Insulin-related traits Consortium (MAGIC) with a large-scale meta-analyses of GWAS for glycaemic phenotypes in persons without diabetes, identified other nine novel associations loci for fasting glucose (*ADCY5, MADD, ADRA2A, CRY2, FADS1*, *GLIS3*, *SLC2A2*, *PROX1* and *C2CD4B*) and one influencing fasting insulin and HOMA-IR near the *IGF1* locus. Evaluating the impact on T2D risk, the MAGIC investigators found novel associations with T2D in additional five loci (*ADCY5*, *PROX1*, *GCK*, *GCKR* and *DGKB-TMEM195*) and replicates the known signals at *TCF7L2* and *SLC30A8* loci [32]. Therefore, the identification of *KCNQ1* in East-Asian samples illustrated how studies from populations of different ancestry might uncover additional T2D loci. Indeed, *KCNQ1* was previously unidentified in samples of European descent. Differences in risk–allele frequency meant that the East-Asian studies were better powered to detect an effect [33]. In support of this, recent studies in populations of Asian ancestry have also found novel association loci reaching genome-wide significance at *PTPRD*, *SRR*, *UBE2E2* and *CDC4A-CDC4B* [33]. Summarizing, to date, 76 T2D susceptibly variants have been identified, explaining only the ~10% of observed familial clustering in Europeans (Figure 3).

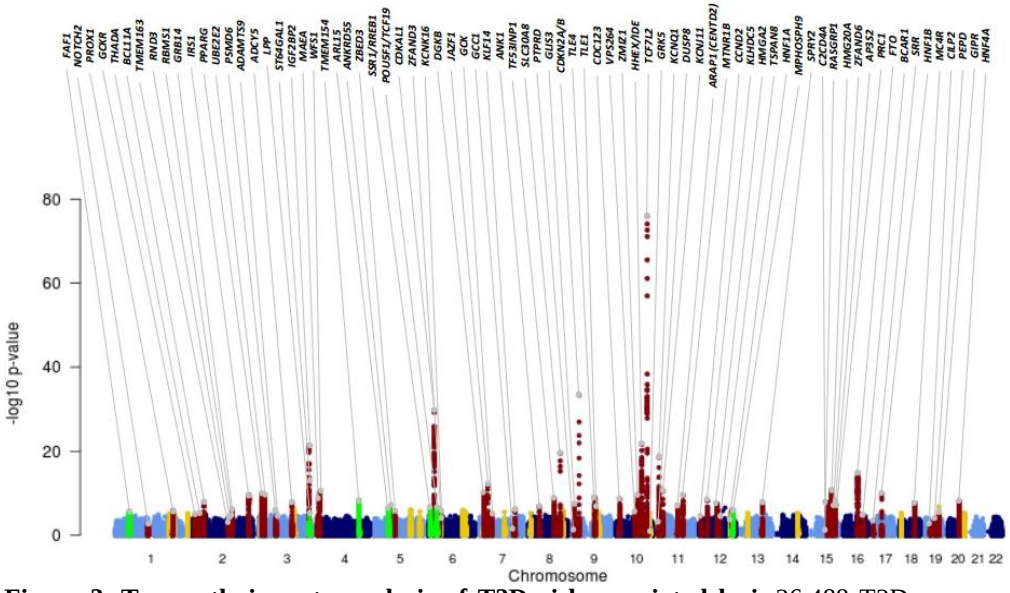

Figure 3. Trans-ethnic meta-analysis of T2D risk-associated loci. 26,488 T2D cases and 83,964 controls from populations of European, East Asian, South Asian, and Mexican and Mexican American ancestry. Nat Genet. 2014 Mar;46(3):234-44.

Alongside T2D risk-associated loci, the noncoding variation in SNPs within introns 1 and 2 of *FTO* (*fat mass and obesity associated*) gene is also the strongest genetic association with risk to polygenic obesity in humans [34]. After the previously reported variants in *FTO* gene, the other strongest association signal mapped 188 kb downstream of *MC4R* (*melanocortin-4 receptor*) gene, mutations of which are the leading cause of monogenic obesity in humans [35]. In the 2009, a meta-analysis of GWAS by the Genetic Investigation of ANthropometric Traits (GIANT) consortium confirmed the *FTO* and *MC4R* genes as BMI associated-loci and identified six additional loci as genome-wide significant: *TMEM18, KCTD15, GNPDA2, SH2B1, MTCH2* and *NEGR1*[36]. Another study by *Thorleifsson et al.* published in the same issue of Nature Genetics replicated their findings confirming known associations to BMI of variants in or near the loci *FTO*, *MC4R*, as well as for the loci *NEGR1*, *TMEM18*, *KCTD15* and *SH2B1* and identified additional two new loci: *ETV5* and *BDNF* [37]. Some obesity-related genes are reported in Table 3 [38]. Thus, substantial efforts have been made to define loci and variants contributing to the individual risk of both T2D and obesity, however, the overall risk explained by genetic variation is very modest.

Nearest gene	*Full gene name*	*Chr*	*Trait*
ADAMTS9	*ADAM metallopeptidase with thrombospondin type 1 motif 9*	3	WHR
BDNF	*Brain-derivedneurotrophicfactor*	11	BMI
C12orf51	*Chromosome 12 openreadingframe 51*	12	WHR
CADM2	*Cell adhesion molecule 2*	3	BMI
CDKAL1	*CDK5 regulatory subunit associated protein 1-like 1*	6	BMI
CPEB4	*Cytoplasmicpolyadenylation element binding protein 4*	5	WHR
DNM3/PIGC	*Dynamin 3 – phosphatidylinositol glycan anchor biosynthesis class C*	1	WHR
ETV5	*Ets variant 5*	3	BMI
FAIM2	*Fas apoptoticinhibitorymolecule 2*	12	BMI
FANCL	*Fanconi anemia complementation group L*	2	BMI
FLJ35779	*POC5 centriolar protein homolog*	5	BMI
FTO	*Fat mass and obesity associated*	16	BMI
GNPDA2	*Glucosamine-6-phosphate deaminase 2*	4	BMI
GP2	*Glycoprotein 2zymogengranulemembrane*	16	BMI
GPRC5B	*G protein-coupled receptor family C group 5 member B*	16	BMI
GRB14	*Growth factor receptor-bound protein 14*	2	WHR
HOXC13	*Homeobox C13*	12	WHR
IRS1	*Insulin receptor substrate 1*	2	Fat %
ITPR2/SSPN	*Inositol 1,4,5trisphosphate receptor type 2/sarcospan Kras oncogeneassociated gene*	12	WHR
KCTD15	*Potassium channel tetramerisation domain containing 15*	19	BMI
KLF9	*Kruppel-like factor 9*	9	BMI
LRP1B	*Low density lipoprotein receptor-related protein 1B*	2	BMI
LRRN6C	*Leucine rich repeat neuronal 6 C*	9	BMI
LY86	*Lymphocyte antigen 86*	6	WHR
LYPLAL1	*Lysophospholipase-like 1*	1	WHR
MAP2K5	*Mitogen-activated protein kinase kinase 5*	15	BMI
MC4R	*Melanocortin 4 receptor*	18	BMI
MRPS22	*Mitochondrialribosomal protein S22*	3	BMI
MSRA	*Methioninesulfoxidereductase A*	8	WAIST
MTCH2	*Mitochondrialcarrier 2*	11	BMI
MTIF3	*Mitochondrialtranslational initiation factor 3*	13	BMI
NEGR1	*Neuronal growth regulator 1*	1	BMI
NFE2L3	*Nuclear factor (erythroid-derived 2)-like 3*	7	WHR
NISCH/STAB1	*Nischarin/stabilin 1*	3	WHR
NRXN3	*Neurexin 3*	14	BMI
NUDT3	*Nudix (nucleoside diphosphate linked moiety X)-type motif 3*	6	BMI
PAX5	*Paired box 5*	9	Fat mass
PCSK1	*Proproteinconvertasesubtilisin/kexin type 1*	5	BMI
PRKD1	*Protein kinase D1*	14	BMI
PTBP2	*Polypyrimidinetractbinding protein 2*	1	BMI
QPCTL/GIPR	*Glutaminyl-peptide cyclotransferase-like/gastric inhibitory polypeptide receptor*	19 19	BMI BMI
POMC/ADCY3	*Proopiomelanocortin/adenylatecyclase3*	2	BMI
RPL27A	*Ribosomal protein L27a*	11	BMI
RSPO3	*R-spondin3*	6	WHR
SEC16B	*SEC16 homolog B*	1	BMI
SH2B1	*SH2B adaptor protein 1*	16	BMI
SLC39A8	*Solute carrier family 39 member 8*	4	BMI
SPRY2	*Sprouty homolog 2*	13	Fat %
TBX15/WARS2	*T-box 15/tryptophanyltRNA synthetase 2 mitochondrial*	1	WHR
TFAP2B	*Transcription factor AP-2 beta (activating enhancer binding protein 2 beta)*	6	BMI
TMEM160	*Transmembrane protein 160*	19	BMI
TMEM18	*Transmembrane protein 18*	2	BMI
TNNI3K	*TNNI3 interactingkinase*	1	BMI
VEGFA	*Vascular endothelial growth factor A*	6	WHR
ZNF608	*Zinc finger protein 608*	5	BMI
ZNRF3-KREMEN1	*Zinc and ring finger 3/kringle containing transmembrane protein 1*	22	WHR

Table 3. SNPs reported to be associated to BMI, waist circumference, waist–hip ratio, fat percentage or fat mass in GWAS. Chr: chromosome, **BMI:** body mass index, **WHR:** waist to hip ratio. Mol Cell Endocrinol. 2014 Jan 25;382(1):740-57.

2.1 Epigenetics

Substantial evidence has brought to light that environmental factors including diet, physical activity, drugs and smoking, affect the phenotype and provide a major contribution to susceptibility to most chronic non communicable diseases [39]. Epigenetics represents the fastest growing research areas in biomedicine, acting at the interface between the genome and environmental factors. It might be broadly defined as the sum of all the mechanisms necessary to unfold the genetic program into development [40]. In the early 1940s, Conrad Waddington linked genetics and developmental biology coining the term epigenetics, defining it as "the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being" [41]. However, the meaning of the word has gradually changed over the following years, and epigenetics is known today as "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence" [42]. Differently from traditional genetics, based on cell lineages and clonal inheritance, epigenetic changes often occur in groups of cells while some epigenetic event is clonal. In addition, genetic changes are, almost by definition, stable, whereas epigenetic changes are plastic events [40]. Epigenetic mechanisms are plastic genomic processes that change genome function under endogenous and exogenous influences [43-44], and may propagate modifications of gene activity from one cell generation to the next [45]. These mechanisms imply chemical modifications of DNA, such as DNA methylation, post-traslational changes in histone proteins altering chromatin conformation, and transcriptional gene silencing mediated by non-coding RNAs (ncRNAs) [46] (Figure 4).

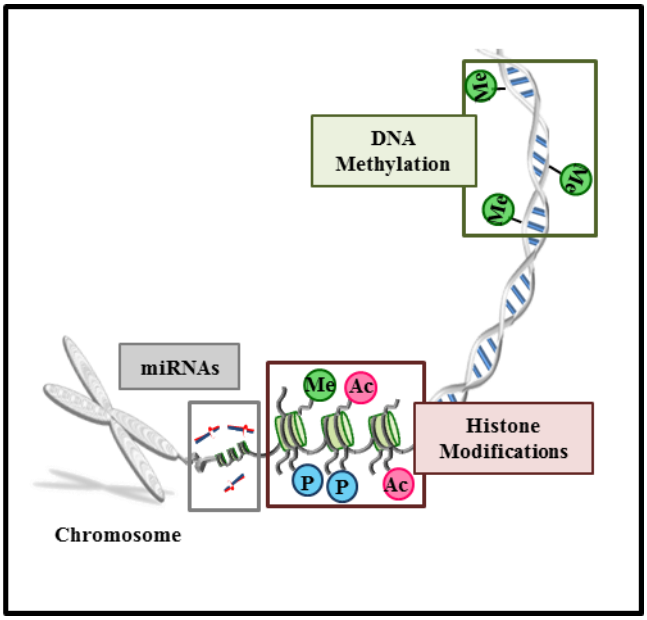

Figure 4. Schematic representation of epigenetic modifications. Epigenetic modifications include DNA methylation, histone modifications and miRNAs. **Ac:** acetylation, **Me:** methylation, **P:** phosphorylation. J Endocrinol Invest. 2016 Oct;39(10):1095-103.

Abnormalities in one or more of these mechanisms can lead to inappropriate expression or silencing of genes, resulting in imbalance of the epigenetic network and may result in metabolic disorders such as T2D and obesity [47-48]. DNA methylation is the best characterized epigenetic mark. In mammals, it is essential during development and is involved in a variety of biological processes, including genomic imprinting and X chromosome inactivation [49]. It has long been recognized as an epigenetic silencing mechanism [50] which preferentially occurs at CpG di-nucleotides that are usually clustered in the CpG islands (CGIs) [51]. Quite often, un-methylated CpG sites (CpGs) at gene promoters create a transcriptionally permissive chromatin state by destabilizing nucleosomes and facilitating the recruitment of transcription factors [52]. On the other hand, dense DNA methylation of CpGs mediates stable long-term gene silencing by direct inhibition of binding of transcription factors or by a combination of events mediated by methyl-CpG binding domain proteins (MBDs) which recruit methylated DNA mediators of chromatin remodeling, such as histone deacetylases (HDACs), or other repressors of gene expression [50, 53-54]. Furthermore, findings over the past ten years have progressively revealed the relevance of ncRNAs in most epigenetically-controlled events. The most extensively studied ncRNAs are the miRNAs, that are critical regulators of post-transcriptional gene expression. In particular, miRNAs are able to suppress the target gene expression binding the 3' Untranslated Region (3'UTR) of the target mRNA Figure 5. Moreover miRNAs are susceptible to epigenetic modulation, indeed, are able to regulate both DNA methylation and histone modifications by controlling the expression of important epigenetic regulators, including DNA methyltransferases (DNMTs) and HDACs thereby impacting on the entire gene expression profile [55].

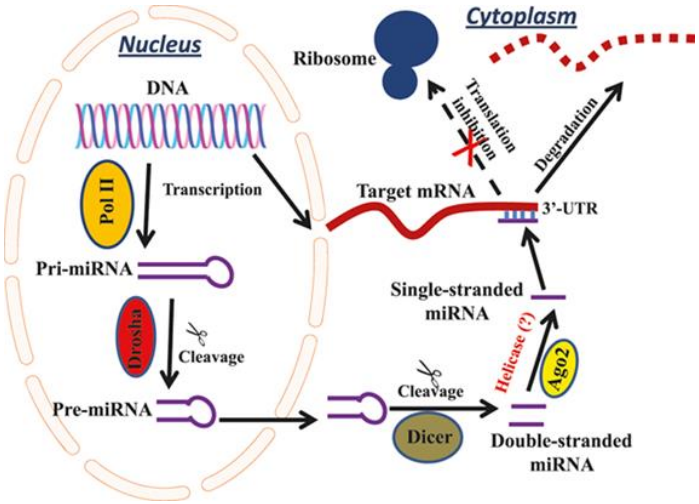

Figure 5. miRNA action. The single-stranded mature miRNAs then act as the core of the RNA-induced silencing (RISK) complex. This complex guides the miRNA to its target sequence located at the 3'-UTR of the target mRNA. Incomplete or complete base-pairing results in degradation of the mRNA or inhibition of translation. Anesthesiology. 2014 Aug;121(2):409-17.

2.2 Epigenetics in T2D and obesity

As previously discussed, T2D and obesity reached epidemic proportions globally [56-57]. Population and family studies have extensively documented the familial aggregation of these diseases [58-63] with more than 175 genetic loci conclusively associated [64] but the impact of these loci, even in combination, on risk is very modest (5-10 % for T2D and ~ 2% for body mass index, BMI), leaving the heritability issue unsolved [65]. Technical limitations might, in part, account for this situation [66]. More likely, inheritance may be explained by epigenetics. Indeed, familial aggregation may reflect not only genetic influences, but also represent the effects of a shared family environment and thus of common environmentally-induced epigenetic modifications [65]. In addition, while not been proved in humans yet, in rodents, environmentally-induced epigenetic modifications can be trans-generationally transmitted to the offspring [67-68]. For instance, environmentally-induced epigenetic modifications may further explain the global epidemics of T2D and obesity, whose exponential rise in the past decades have been related to rapid cultural and social changes, such as socio-economic status, dietary changes, physical inactivity and unhealthy behaviours, all of which tend to cluster in family groups [56, 69]. Finally, epigenetics may help to understand the identical twin discordance for obesity and T2D [70-71]. For example, the concordance rates for T2D among monozygotic twins is only ~ 70 % [74]. In these metabolic disorders the incomplete concordance may be, in part, due to stochastic or environmentally determined epigenetic modifications that change over the lifetime and is responsible for the phenotypic differences and susceptibility to disease. Thus, epigenetic processes may therefore contribute to the development of T2D and obesity and mediate the effects of environmental exposure on risk [46, 73].

2.2.1 Epigenetic event in T2D

To date substantial evidence indicates that environmentally-induced epigenetic changes contribute to diabetes prevalence (Table 4). *Ling et al.* have recently demonstrated that the promoter of the transcriptional co-activator *Peroxisome proliferator activated receptor gamma coactivator-1 alpha* (*PGC1-α*) gene, mainly involved in mitochondrial function, is highly methylated in pancreatic islets obtained from diabetic patients compared with non-diabetic controls [74]. Additionally, *Barrès et al*., have shown that the hyper-methylation of the *PGC-1α* promoter occurs even in the skeletal muscle from T2 diabetic subjects compared with normal glucose-tolerant (NGT) individuals. *PGC-1α* hyper-methylation negatively correlates with its mRNA expression in these subjects [75]. In addition, the exposure of primary human skeletal muscle cells from NGT individuals to external factors, such as free FFAs and tumor necrosis factor-alpha (TNF-α) directly and acutely alters the methylation status of *PGC-1α* promoter. These findings illustrate how alterations in the extracellular milieu may predispose to T2D by inducing DNA methylation changes [75]. Furthermore, a genome-wide DNA methylation analysis of skeletal muscle

from obese subjects before and after bariatric surgery provides evidence that the promoter methylation of *PGC-1α* is altered by obesity and restored after weight loss. DNA methylation inversely correlates to BMI, leptin, triglyceride and insulin levels in these subjects, which supports the role of DNA methylation in the physiological control of *PGC-1α* gene transcription [75-76]. More recently, a genome-wide analysis of differentially methylated sites in genomic regions associated to T2D has revealed that the *FTO* gene is hypo-methylated in a CpG site within the first intron in T2 diabetics compared with control subjects in human peripheral blood. The T2D predictive power of this mark is significantly greater than all genetic variants so far described [77]. In the same investigation *Toperoff et al*. have also prospectively established, that, in an independent cohort hypo-methylation at the *FTO* intron is observed in young subjects that later progress to T2D. This further finding provides evidence that methylation changes may predispose to T2D and deserve to be considered and further investigated as T2D marker. Even miRNAs have been associated to T2D onset and have been involved in the regulation of multiple pathways including insulin signaling and release [78]. For instance, the miRNA-375 has been recently identified to have a specific role in the pancreatic islets [79]. Its overexpression in the rat insulinoma INS-1E cells reduces the glucose-induced insulin secretion, while its inhibition results in an increased insulin release [79]. The up-regulation of the miR-222, miR-29a, and miR-335 have instead a functional and biological relevance in the WAT of diabetic rats [80]. Furthermore, through a microarray based approach the up-regulation of the miR-335 has been also identified in the liver of diabetic rats contributing to the fatty liver disease and associated phenotypes [81]. Altogether these findings provide further evidence that both DNA methylation and miRNAs expression changes might predispose to T2D and its related phenotypes.

2.2.2 Epigenetic event in obesity control

It has been extensively documented both in humans and animal models that a relationship exists between obesity and the epigenetic regulation of genes involved in the control of food intake (Table 4) [68, 82-86]. In humans, DNA methylation of the *Pre-proopiomelanocortin (POMC)* gene which encodes the anorexigenic hormone α-MSH produced by neurons of hypothalamic arcuate nucleus has been associated with the individual risk of childhood obesity [87]. In particular, using peripheral blood cells, *Kuenen et al*. have found hyper-methylation at the Intron 2/Exon 3 boundary of the *POMC* gene in obese compared with normal weight children. In particular, in these obese children, the *Alu* elements, which are known to influence methylation in their genomic proximity at the Intron 2, trigger a default state methylation at the Intron 2/Exon 3 boundary, interfering with binding of the histone acetyltransferase/transcriptional coactivator p300 and reducing *POMC* expression [87]. In addition, several studies suggest a critical role of epigenetic marks also as predictors of susceptibility to obesity and metabolic disease in

humans and animal models [88-89]. A further example of this concept in humans has been provided by studies on the *retinoid X receptor-alpha* (*RXRA*) gene. *Godfrey at al.,* designed a perinatal epigenetic analysis of the methylation status of CpG sites at the promoters of 78 selected candidate genes in DNA from umbilical cord tissue of children who were assessed for adiposity at age 6 and 9 years. These authors have established that the variation of adiposity and the onset of obesity in pre-pubertal children was associated with the specific hyper-methylation of a CpG site at the RXRA chr9:1363558885+ at birth [89]. Furthermore, in the same population, it was demonstrated that this neonatal epigenetic mark was associated with lower maternal carbohydrate intake in pregnancy first trimester, providing a further example of how epigenetic processes may link the early prenatal life with the predisposition to obesity and other phenotypic outcomes [89]. Dysregulated post-transcriptional gene silencing contributes to the development of obesity-induced insulin resistance as well. For example, a microarray analysis on serum from young adult has highlighted that elevated levels of the circulating miRNA-122 are positively associated with obesity and insulin resistance and that the increase of this miRNA correlates with BMI, triglycerides, HDL-cholesterol and homeostasis model assessment of insulin resistance (HOMA-Index) in humans [90]. Additionally, the overexpression of miRNA-143 inhibits the insulin-stimulated Pkb activation and impairs glucose metabolism in the liver of obese mice [91]. Furthermore, miRNA-143 is also involved in adipocyte biology. Indeed, the transfection of an antisense miRNA-143 oligonucleotide in cultured human preadipocytes inhibits adipocyte differentiation of about 40% by reducing the expression of specific markers such as the *Glucose transporter 4* (*GLUT4*), the *Fatty acid-binding protein aP2*, and the *Peroxisome proliferator-activated receptor γ2* (*PPARγ2*) [92]. On the other hand, the ectopically expression of the miR-143 causes the opposite effect by upregulating the same adipogenesis markers. [93].

Genes	***Regions***	***Epigenetic modification***	***Phenotypes***	***Tissues/Cells***
PGC1α	Promoter	↑ DNA Methylation	T2D	Pancreatic islets, Skeletal muscle
PDX-1	Promoter	↑ DNA Methylation	T2D	Pancreatic islets
FTO	Intron 1	↓ DNA Methylation	T2D	PBLs
POMC	Intron 2-Exon 3	↑ DNA Methylation	Obesity	PBLs
RXRA	Promoter	↑ DNA Methylation	Obesity	Umbilical cord
Leptin	Promoter	↑ DNA Methylation	Obesity	Adipose tissue

Table 4. DNA methylation in T2D and Obesity T2D: Type 2 Diabetes, **PBLs:** Peripheral Blood Leucocytes, ***PGC1α:*** *Peroxisome Proliferator Activated Receptor Gamma Coactivator-1 alpha,* ***PDX-1:*** *Pancreatic duodenal homeobox1,* ***FTO:*** *Fat mass and Obesity-associated,* ***POMC:*** *Preproopiomelanocortin,* ***RXRA:*** *Retinoid X receptor-alpha.* J Endocrinol Invest. 2016 Oct;39(10):1095-103.

3.1 Approaches for the identification of disease risk-associated genes

Understanding the genetic basis of susceptibility to disease has become increasingly important for research. The main approaches for the identification of novel risk-associated genes are basically two: candidate gene study and GWAS; both approaches have a combination of benefits and drawbacks [94]. Candidate gene study is the most common method for associating human genetic variations with phenotypes. Historically, association studies to detect alleles conferring increased or decreased risk to common diseases have employed markers in candidate genes, or in all the genes belonging to a biological pathway or having a similar biological function [95]. These approaches are relatively cheap and quick to perform and the success of the analysis depends on the correct choice of which genes/pathways to study. Therefore, a priori hypothesis about biological function is definitely required [96]. Candidate genes can be identified by several methods including GWAS and linkage studies. A combination of linkage mapping and candidate gene study has been the most successful method for identifying disease genes to date [97]. On the other hand, the GWAS represents a method for the identification of genes implicated in several human diseases. It has been made possible by the development of high-density genotyping arrays that leverage the knowledge generated from the International HapMap project [98]. This approach involves a rapid scanning of markers across genomes of many people to find genetic variations associated with several diseases. The idea that common diseases have a different underlying genetic architecture than rare disorders, coupled with the discovery of several susceptibility variants for common disease with high minor allele frequency (including alleles of PPARγ gene for T2D), led to the development of the common variant (CV) hypothesis [98]. This hypothesis states that common disorders are likely influenced by genetic variation and are common in the population. Therefore, over the last five years, the CV hypothesis has been tested for a variety of common diseases, and while much of the heritability for these conditions is not yet explained, common alleles certainly play a role in susceptibility [98]. Thus, combined with advances in technology as the next generation sequencing approaches (NGS), genome-wide genotyping can now be readily carried out to accurately 'tag' the vast majority of the diversity in the genome. However, some drawbacks are to be considered. Indeed, for GWAS approach, a large cohort size is an important factor to ensure sufficient statistical power. Thus, a large study population is a necessary pre-requisite and the results needs replication in independent samples. GWAS are also able to identify a specific location and not complete genes, and are able to detects only common variants (>5%) as well [98]. Furthermore, the interpretation of the data is not a simple matter and appropriate statistical methods are necessary to reduce the risk of multiple false positive results. Although, relatively few studies have explored the epigenetic characterization of humans T2D or obesity risk, in Table 5 are summarized the methylation status of selected CpG sites in candidate genes and in the epigenome-wide association study (EWAS) [66].

Candidate genes/EWAS	***Phenotypes***	***Tissue***	***Ref.***
CCL2	T2D	Peripheral blood mononuclear cells	*Liu (2011)*
INS	T2D	Pancreatic islets	*Yang (2011)*
PDX1	T2D	Pancreatic isle	*Yang (2012)*
PPARGC1A	T2D	Pancreatic islets	*Ling (2008)*
EWAS	T2D	Skeletal muscle	*Barres (2009)*
EWAS	T2D	Whole blood	*Bell (2010)*
EWAS	T2D	Whole blood	*Toperoff (2012)*
EWAS	T2D	Pancreatic islets	*Volkmar (2012)*
ALOX12, ALPL, BCL2A1, CASP10, CAV1,CCL3,CD9, CDKN1C, DSC2, EPHA1, EVI2A, HLA, IRF5,KRT1, LCN2, MLLT4, MMP9, MPL, NID1, NKX31,PMP22, S100A12, TAL1, VIM	BMI, fat mass, and lean mass	Umbilical cord blood	*Relton (2012)*
KCNQ1OT1, H19, IGF2, GRB10,MEST,SNRPN,GNAS	BMI (discordance in twins)	Saliva	*Souren (2011)*
MCHR1	BMI	Whole blood	*Stepanow (2011)*
POMC	Obesity	Whole blood	*Kuehnen (2012)*
IL8, NOS3, PIK3CD, RXRA, SOD1	Fat mass and % fat mass	Umbilical cord tissue	*Godfrey (2011)*
SLC6A4	BMI, weight, and waist circumference	Peripheral blood leukocytes	*Zhao (2012)*
TACSTD2 EWAS	Fat mass BMI	Whole blood Lymphocytes	*Groom (2012)* *Feinberg (2010)*
EWAS	Obesity	Peripheral blood leukocytes	*Wang (2010)*
EWAS	Obesity	Whole blood	*Almen (2012)*

Table 5. DNA methylation candidate gene and EWAS for T2D and Obesity. BMI: body mass index, **EWAS:** epigenome-wide association study, **T2D:** Type 2 Diabetes. Clin Pharmacol Ther. 2012 Dec;92(6):707-15.

4.1 GWAS analysis identified *Ankrd26* as a candidate gene for the study of obesity and T2D risk

The *Ankyrin repeat domain 26 (ANKRD26)* is a newly described gene located at 10p12, a locus identified with some forms of hereditary obesity in humans. The gene encodes a ~190 kDa protein that is highly abundant in the hypothalamus and other regions of the brain known to play a key role in regulation of feeding behavior, as well as in many tissues and organs, including insulin target tissues like liver, skeletal muscle and withe AT (WAT). It has been demonstrated that *Ankrd26* gene takes part in the development of both obesity and T2D in mice. Indeed, mice with a partial inactivation of this gene show an obese phenotype which results from a marked hyperphagia rather than a reduction of the energy expenditure and activity [99]. Furthermore, when deleted at its C-terminus, Ankrd26 leads to excessive food intake and obesity due to severe region-specific changes in primary cilia in the brain [100]. In addition to its function in appetite control, the *Ankrd26* gene has a role in the regulation of adipocyte differentiation in mouse embryonic fibroblasts and in 3T3-L1 cells [101-102]. Beside this, through methylated DNA immuno-precipitation sequencing (MeDIP-seq) approach we have recently identified *Ankrd26* as a gene sensitive to nutrition-induced epigenetic changes. We showed that hyper-methylation of the *Ankrd26* promoter occurs in VAT from obese mice upon prolonged high fat diet (HFD) feeding compared to age- and sex-matched standard chow diet (STD) fed mice and directly interferes with the binding of the histone acetyltransferase/transcriptional coactivator p300 to this same region. These events result in promotion of chromatin condensation with consequent down regulation of the *Ankrd26* gene expression (Figure 6) [103]. We have further reveled that *Ankrd26* silencing alters secretion of pro-inflammatory adipokines *in vitro*, indicating that the epigenetic silencing of *Ankrd26* gene might be one of the mechanisms responsible for VAT inflammation in response to HFD [103]. Additionally, computational data from a genome-wide DNA methylation analysis in scAT revealed that *ANKRD26* gene is included in a list of 2825 genes where both DNA methylation and mRNA expression levels significantly correlate with BMI in humans [104]. These observations paved the way to supports the hypothesis that the epigenetic regulation of *ANKRD26* gene may occur in humans, as well as in mice, and may represent a pathogenic mechanism by which environmental exposures to nutrients contribute to disease susceptibility through epigenetic modifications.

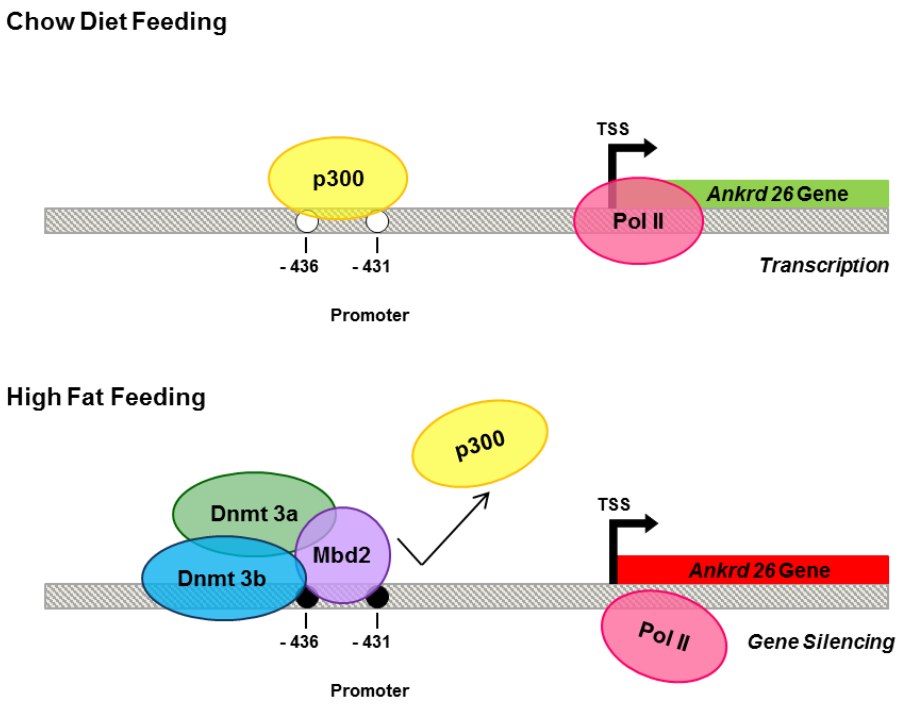

Figure 6. Schematic representation of the epigenetic regulation at the *Ankrd26* promoter gene in mice. The strong reduction of the *Ankrd26* mRNA expression in VAT of HFD fed mice is due to an epigenetic regulation that involve the hyper-methylation of two specific CpG sites by Dnmts 3a and 3b, the bind of Mbd2 to the methylated CpG sites that in turn crowd out the HAT p300 recruitment to the *Ankrd26* promoter, resulting in hypo-acetylation of histone H4 and reduced binding of RNA Pol II on the TSS of the *Ankrd26* gene.

Aim of the study

This thesis concerns the investigation of the "Epigenetic profiling" in individuals at high risk of Type 2 Diabetes (T2D), a common metabolic disorder, which now reached epidemic proportions globally.
To date, the known genetic loci associated with this phenotype do not account either for the current epidemics of the pathology or for the family transmission. However, recent clinical, epidemiological and experimental evidence indicates that environmental factors have an extraordinary impact on the natural history of this disorder. Furthermore, different studies have also documented that the familial aggregation typical of T2D reflects not only genetic influences, but the effects of a shared family environment too, and that environmental hits bring to epigenetic changes, which alter the function of genes affecting disease susceptibility.
Thus, epigenetics may in part explain the environmental origin as well as the familial aggregation of T2D and fill the genetic lack.
In this context, I have addressed with two separate approaches whether: i) changes of the DNA methylation status of the *ANKRD26* gene occur in human obesity; and ii) altered miRNA expression may precede adipocyte hypertrophy in First Degree Relatives of T2D patients.
For the first point, I analyzed using a candidate gene study the expression of the *ANKRD26* gene and the DNA methylation status of its promoter in lean and obese subjects, and I also evaluated the correlation between these two events in different obesity sub-phenotypes.
For the second point, I analyzed by an EWAS approach the epigenetic profiling of lean euglycaemic first degree relatives of Type 2 diabetic subjects (T2D-FDRs) looking at the correlation between miRNA expression and adipocyte hypertrophy. Furthermore, I also looked for specific target genes that may be regulated by changes of the miRNA expression and may be responsible for adipocyte hypertrophy in T2D-FDRs.

Materials and Methods

Subjects

For the candidate gene study, 14 lean and 20 severely obese subjects were recruited at the "Federico II" University of Naples. The complete clinical characteristics of the subjects that participated in this study are given in Table 6, Table 7 and Table 8. For the genome wide study, 11 lean individuals without familiarity for T2D and 9 lean T2D-FRDs were recruited at the Sahlgrenska University Hospital of Göteborg. The characteristics of subjects are detailed in Table 10. Leanness was defined as BMI < 25 kg/m^2, while severely obesity as BMI ≥ 35 kg/m^2. The 75-g oral glucose tolerance test (OGTT) was performed to assess the glucose tolerance and the diagnosis of diabetes has been carried out according to the American Diabetes Association (ADA) criteria [105]. The degree of insulin sensitivity was measured by the euglycemic-hyperinsulinemic clamp over 120 min and expressed as glucose infusion rate (GIR) normalized by total body weight. Participants with metabolic and endocrine disorders, inflammatory diseases, previous or current malignancies, and/or treated with drugs able to interfere with the epigenome were excluded from the study. Each active ingredient has been evaluated using the The Comparative Toxicogenomics Database (CTD). Both the studies adhered to the Declaration of Helsinki, the gene candidate study has been reviewed and approved by the Ethics Committee of the "Federico II" University of Naples (Ethics Approval Number: No. 225_2013), while the EWAS were approved by the local Ethical Committees at the Sahlgrenska Academy and the Karolinska Institute. Informed consent was obtained from all of enrolled individuals.

Stromal vascular fraction cells isolation

Adipose tissue biopsies were obtained from the abdominal subcutaneous adipose tissue around the umbilicus. Following careful dissection, tissues were digested with 0.8 mg/ml collagenase in Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 (1:1) for 45 min at 37°C. The solution were then filtered through a 250-m nylon mesh. The medium was collected and centrifuged for 10 min at 200g. The cell pellet containing the SVFs was washed twice and the erythrocytes were lysed with 155 mmol/l NH4Cl for 5 min before seeding the cells in a 55-cm^2 petri dish. After 3 days, when the cells had started to proliferate, the progenitor or inflammatory cells were isolated with magnetic immune separation. The remaining cells were then cultured at 37°C with DMEM and F12 (1:1) with 10% fetal bovine serum (FBS), 2 mmol/l glutamine, 100 units/ml penicillin, and 100 μg/ml streptavidin. After 2 weeks cells were trypsinized and any remaining inflammatory cells removed by magnetic immune separation of CD14 and CD45 positive cells [23].

Sampling

Tissues from obese subjects were homogenized by TissueLyser LT (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Total RNA and genomic DNA were isolated using the AllPrep DNA/RNA/miRNA Universal kit (Qiagen). Peripheral blood leucocytes (PBLs) from lean and obese subjects were isolated from whole blood. Samples were incubated in ice for 15 min with 5 volume of erythrocyte lysis buffer ($KHCO_3$ 10 mM, NH_4Cl 155 mM, EDTA 0.1 mM) and centrifuged at 400 x g for 10 min. Total RNA and genomic DNA were isolated as above. SVFs were lysate through a 20-gauge needle and fitted to an RNase-free syringe. Total RNA and genomic DNA were isolated as above

RNA sequencing and quantitative Real Time PCR (qPCR)

Total RNA was isolated from PBLs and SVFs as above. cDNA synthesis and qPCR were performed as described [106]. For RNA sequencing 5 µg of pooled total RNA from SVFs of each one of the 4 experimental groups (2 group of T2D-FDR and 2 Control groups) was analyzed. The sequencing was carryout by Illumina's HiSeq technology generating 8G clean 90bp paired-end reads per sample.

Primers sequences: hs28S F: 5'-cccagtgctctgaatgtcaa-3', hs28S R: 5'-agtgggaatctcgttcatcc-3'; hsANKRD26 F: 5'-gtatgctagtagtggtcctgc-3', hsANKRD26 R: 5'-gtaggccttccttcatcctcat-3'; hsRPL13A F: 5'-ctttccgctcggctgttttc-3', hsRPL13A R: 5'-gccttacgtctgcggatctt-3'; hsPTPRD F: 5'-tttacacgaacacccgttga-3', hsPTPRD R: 5'-cggagtccgtaagggttgta-3'; hsIGF2 F: 5'-ggaagtgagcaaaactgccg-3', hsIGF2 R: 5'-aagatgctgctgtgcttcct-3'; hsINMT F: 5'-aattcgcctgtgagctggaa-3', hsINMT R: 5'-aggtggacatcgcacttgag-3'; hsMXRA5 F: 3'-tccaccagagcagctcaaag-5', hsMXRA5 R: 5'-acacagtgtctgtctcagcg-3'; hsOXTR F: 5'-tggcataagtgctctgctcc-3', hsOXTR R: 5'-ccaaggaggggagggataca-3'; hsRGS4 F: 5'- aggcagagggatgaaatgcc-3', hsRGS4 R: 5'-agtgactcacacatggcagg-3'; hsSYNPO2L F: 5'-gatggtggaagggaggtgtg-3', hsSYNPO2L R: 5'-gatggggtgggactgaaagg-3'; hsPRELP F: 5'-cctccccacccccaataggat-3', R: 5'- ctgcctctcctctcagctct-3'.

miRNA reverse transcription miRNAs sequencing and qPCR

Total RNA was isolated from SVFs. Than 3 µg of pooled total RNA enriched in small RNA from each one of the 4 experimental groups (2 group of T2D-FDR and 2 Control groups) was used for the miRNA sequencing. The miRNome was analyzed by NGS using Miseq™ Platform (Illumina®). In a single run, more than 1 million reads were generated for each pool and the miRNA sequencing data were aligned to human mature microRNA sequences (from miRBase Version 21). The differential expression of miRNAs was then validated by qPCR. After quantification with NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA) total RNA was reverse transcribed using the miScript II RT Kit (QIAGEN) and analyzed using the miScript SYBR Green PCR Kit (QIAGEN) with specific miScript Primer

Assays. The levels of all differential expressed miRNAs were quantified as absolute expression units and 95snRNA was used as housekeeping small RNA. Specific primers used for amplification were purchased from QIAGEN: MS0003164 hs_miR-23a*_2 miScript Primer Assay; MS00008932 hs_miR-193a-5p_1 miScript Primer Assay, MS00008939 hs_miR-193b*_1 miScript Primer Assay, MS00033726 hs_SNORD95_11 miScript Primer Assay.

DNA methylation analysis by bisulfite conversion

Genomic DNA from PBLs was prepared as described above. Bisulfite treatment of 350 ng of genomic DNA for each sample was converted with the EZ DNA Methylation Kit (Zymo Research, Orange, CA), following the manufacturer's instructions. For the analysis the *ANKRD26* promoter was divided into 5 regions: Site 1 (S1), -786/-722 bp; Site 2 (S2), -716/-370 bp; Site 3 (S3), -349/-48 bp; Site 4 (S4), -68/+147 bp and Site 5 (S5), +134/+390 bp from the transcription start site (TSS). Bisulfite-converted genomic DNA was amplified by PCR using specific primers for each site. The PCR fragments were then cloned into the pGEM T-Easy vector system (Promega, Madison, WI). To determine methylation status, 10 clones for each sample were sequenced on AB 3500 genetic analyzer (Life Technologies) and the percentage of methylation was calculated using the following formula: $(CpG_{methylated} / CpG_{total}) \times 100$. Primers sequences:
ANKRD26 sub-region S1 F: 5'-gtaatttttgttgagattttatttga-3', ANKRD26 sub-region S1 R: 5'-actacaatctccacctcctaaactc-3', ANKRD26 sub-region S2 F: 5'-agtttaggaggtggagattgtagtg-3', ANKRD26 sub-region S2 F: 5'-acaaatacaacaacaaaaaacacaaa-3', ANKRD26 sub-region S3 F: 5'-gtatttaaagggatatggaaggg-3', ANKRD26 sub-region S3 R: 5'-cccaataatcaaatatactccatac-3', ANKRD26 sub-region S4 F: 5'-tggagtatatttgattattgggtttt-3', ANKRD26 sub-region S4 R: 5'-aacttcaaaaacacctcatatctctct-3', ANKRD26 sub-region S5 F: 5'-agagagatatgaggtgtttttgaagtt-3', ANKRD26 sub-region S5 R: 5'-caaaccattcttcctaaacaaaaaa-3'.

Statistical procedures

Data are expressed as mean ± SD. Comparison between groups were performed using Student's t-test or the one-way analysis of variance. Correlation between two variables was calculated using the parametric Pearson r-test. Pathway analysis was performed using PANTHER™ (Protein ANalysis THrough Evolutionary Reationships) (http://pantherdb.org). For all the procedures, $p<0.05$ was considered statistically significant.

Results and discussion

To investigate and establish the "Epigenetic profiling" of individuals at high risk of Type 2 Diabetes (T2D), during my PhD program I have simultaneously performed two distinct investigations. The first one was a candidate gene analysis performed in lean and obese individuals; while the second one was an Epigenome Wide analysis performed in a population of first degree relatives of T2D patients.

Specific CpGs hyper-methylation relates to ANKRD26 gene down-regulation in human obesity

Concerning the first study, as I mentioned before, *Ankrd26* was identified as a gene sensitive to nutrition/obesity-induced epigenetic changes in mice. Indeed, in mice HFD feeding/obesity causes hyper-methylation of the *Ankrd26* promoter in VAT depots. This increased CpGs methylation in turn, directly interferes with the binding of the histone acetyltransferase/transcriptional coactivator p300 resulting in *Ankrd26* gene down regulation.

In this context, I investigated whether down-regulation of the *ANKRD26* gene expression and increased DNA methylation of the *ANKRD26* promoter relate to body mass even in humans and predominantly occur in obese individuals. Also, I evaluated the correlation between these two events in different obesity sub-phenotypes.

I initially began to address these questions by measuring the *ANKRD26* mRNA levels by qPCR in VAT biopsies from 10 subjects with wide differences of BMI (range: 35-65 kg/m^2). VAT is indeed in humans a fat depot strongly associated with metabolic abnormalities [107], and the *Ankrd26* gene expression is epigenetically altered in this tissue in obese mice [103]. The clinical characteristics of the analyzed individuals are detailed in Table 6. In these subjects, the expression of VAT *ANKRD26* gene negatively correlates to BMI (n=10; r=-0.770; p<0.01) (Figure 7), indicating that the VAT *ANKRD26* down-regulation is associated with increased body mass in humans.

n	10
Sex	***4M\6F***
Age, years	37.5 ±5.7
BMI, Kg/m^2	42.2 ± 4.6
fb-glucose, mg/dL	84.4 ± 5.6
fb-triglycerides, mg/dL	128.8 ± 56.0
fb-total cholesterol, mg/dL	190.2 ± 36.8
HDL, mg/dL	48.8 ±10.3
LDL, mg/dL	125.6 ± 30.2

Table 6. Metabolic parameters of 10 subjects. BMI: Body Mass Index, **fb:** fasting blood, **HDL:** High Density Lipoprotein, **LDL:** Low Density Lipoprotein.

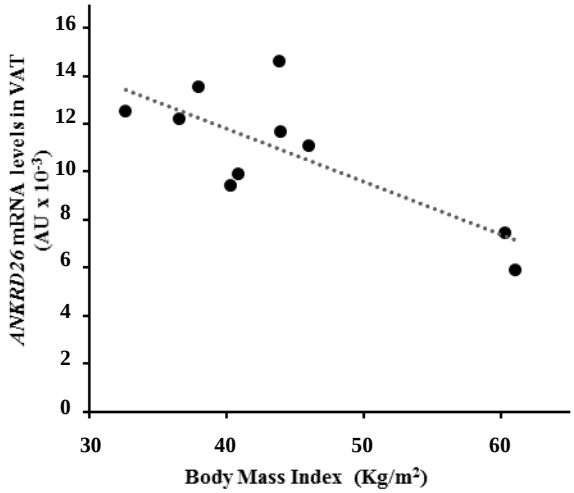

Figure 7. Correlation analysis between VAT *ANKRD26* gene expression and BMI. n=10; r=-0.770; $p<0.01$.

Therefore, to expand and improve the feasibility of this study, I measured the *ANKRD26* mRNA levels in the Peripheral Blood Leucocytes (PBLs) of the same subjects. Circulating biomarkers compared to tissue biomarkers give indeed the advantages of being more readily accessible, minimally invasive and easily utilized for follow-up after treatment modalities [108]. Interestingly, a positive correlation was found between VAT and PBLs *ANKRD26* mRNA levels (n=10; r=0.8021; $p<0.01$) indicating that PBLs *ANKRD26* gene expression relates to the VAT *ANKRD26* gene expression. This suggests that, although PBLs do not represent a system where *ANKRD26* function has been already described, blood samples represent a valid non-invasive proxy system for the study of the relationship between body mass and the *ANKRD26* gene expression (Figure 8). These data, thus, paved the way for the use of PBLs instead of VAT biopsies in the following analysis.

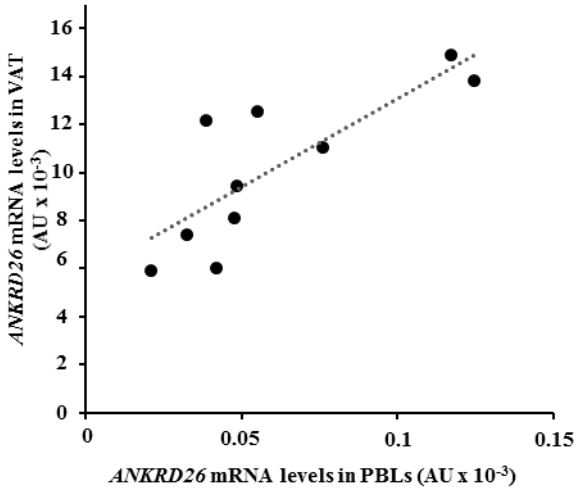

Figure 8. Correlation analysis between VAT *ANKRD26* mRNA levels and PBLs *ANKRD26* mRNA levels. n=10; r=0.8021; $p<0.01$.

Based on the previous results, I thus determined whether the *ANKRD26* gene expression might be indicative of leanness or obesity. To address the last question, I extended my study to a population of 34 subjects, including lean and obese individuals. The clinical characteristics of the lean and obese groups are detailed in Table 7. While there were no significant differences in age, obese subjects had a significant increase in BMI ($p<0.001$), triglycerides ($p<0.001$), low density lipoprotein (LDL) ($p<0.001$) and a significant decrease in high density lipoprotein (HDL) ($p<0.001$) compared with lean individuals.

	Lean	***Obese***
n	14	20
Sex	7M/7F	9M/11F
Age, years	35.2 ± 4.6	38.8± 7.2
BMI, Kg/m²	21.8 ± 2.1	44.8 ± 6.3***
fb-glucose, mg/dL	85.7 ± 6.4	99.5 ± 40.6
fb-triglycerides, mg/dL	52.8 ± 13.5	132.5 ± 46.9***
fb-total cholesterol, mg/dL	166.0 ± 16.8	195.2 ± 37.3
HDL, mg/dL	81.0 ± 9.8	47.0 ± 9.3***
LDL, mg/dL	74.3 ± 10.5	127.3± 30.4***

Table 7. Metabolic parameters of the lean and obese groups. BMI: Body Mass Index, **fb:** fasting blood, **HDL:** High Density Lipoprotein, **LDL:** Low Density Lipoprotein. Values are mean ± SD. Significances (by t-test) were calculated between the two groups. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

The analysis of the *ANKRD26* mRNA levels in the PBLs of 14 lean and 20 severely obese selected subjects further proved that the expression of the *ANKRD26* gene in PBLs negatively correlates to BMI (n=34; r=-0.436; $p<0.01$) (Figure 9a), and revealed that *ANKRD26* mRNA is reduced by about 25% in the obese group compared with the lean control group (lean group *ANKRD26* mRNA: 0.063 ± 0.040 AU x 10^{-3}; obese group *ANKRD26* mRNA: 0.034 ± 0.012 AU x 10^{-3}; $p<0.01$) (Figure 9b). These data are consistent with our previous findings in mice and support the hypothesis that the down-regulation of the *ANKRD26* mRNA levels not only negatively correlates with BMI in humans but it is a common abnormality in obese individuals and may represent a pathogenic mechanism by which the increase of body mass contribute to disease susceptibility.

As previously mentioned, obese individuals can be classified based on their metabolic parameters in healthy and unhealthy obese. Thus, I searched for phenotype differences determining the obesity-related *ANKRD26* gene down-regulation. According to the ADA criteria [105], I classified the previous analyzed obese population based on glucose tolerance in three categories: normo-glucose tolerant (NGT; n=10), impaired glucose tolerant (IGT; n=5) and T2D (n=5) individuals. The characteristic of the three obese groups are reported in Table 8.

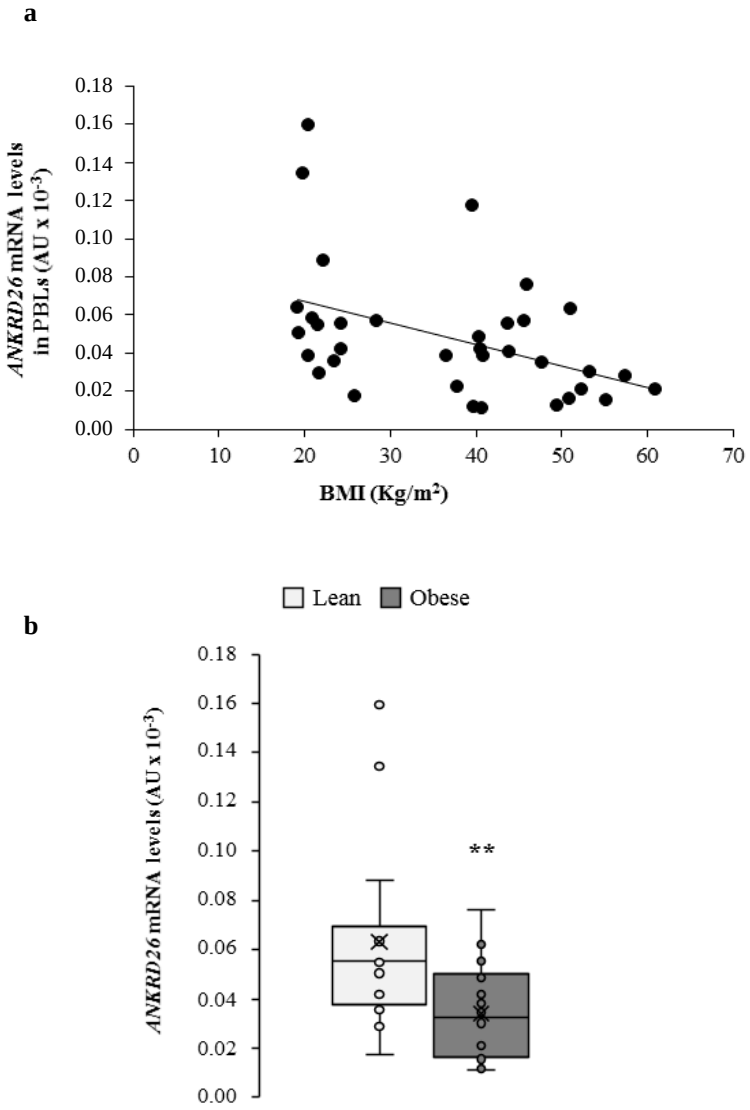

Figure 9. *ANKRD26* mRNA levels in PBLs of lean and obese subjects. a) Correlation analysis of PBLs *ANKRD26* gene expression in relation to BMI. n=34; r=-0.436; $p<0.01$; **b)** *ANKRD26* mRNA levels in 14 lean and 20 obese subjects. Values are mean ± SD. Significances (by t-test) were calculated between lean and obese group. $^{**}p<0.01$.

As expected, the T2D obese subjects have increased levels of the fasting glycaemia ($p<0.05$) and the glycated hemoglobin (HbA_{1c}) ($p<0.001$), and are less sensitive to insulin and less tolerant to glucose, as shown by the higher value of the homeostatic model assessment of insulin resistance (HOMA-IR) ($p<0.05$) and the Oral Glucose Tolerance Test (OGTT) after 2h ($p<0.001$), respectively, compared with both the NGT and the IGT obese subjects; while the IGT obese individuals show increased HbA_{1c} ($p<0.001$) and reduced glucose tolerance by OGTT ($p<0.001$) compared with the NGT obese subjects. No significant differences were, instead, found in fasting blood glucose levels and on the sensitivity to insulin by HOMA-IR among the IGT and NGT obese groups.

	Obese Subjects		
	NGT	**IGT**	**T2D**
n	10	5	5
Sex	4M/6F	3M/2F	2M/3F
Fb-Glucose, mg/dL	84.4 ± 5.5	92.5 ± 39.6	135.4 ± 62.9$^{*,\#}$
HOMA – IR	3.1 ± 1.6	3.9 ± 0.8	7.5 ± 4.6$^{*,\#}$
Hba$_{1c}$, %	5.5 ± 0.1	5.7 ± 0.2***	6.6 ± 0.6$^{***,\#\#\#}$
OGTT p-Glucose 2h, mg/dL	119.3 ± 12.8	145.0 ± 2.6***	281.9 ± 82.0$^{***,\#\#\#}$

Table 8. Classification of the obese population. NGT: Normal glucose tolerant, **IGT:** Impaired glucose tolerant, **T2D:** Type 2 Diabetic, **HOMA-IR:** Homeostatic Model Assessment of Insulin Resistance, **HbA_{1c}:** Glycated Hemoglobin, **OGTT:** Oral Glucose Tolerance Test. Values are mean ± SD. Significances (by t-test) were calculated between T2D and IGT groups Vs. NGT group $*p<0.05$,$***p<0.001$ and between T2D group Vs. IGT group $^{\#}$ $p<0.05$, $^{\#\#\#}p<0.001$.

The analysis of the *ANKRD26* mRNA levels in the PBLs of the obese population classified as above revealed that no significant differences were found in *ANKRD26* gene expression within the NGT, IGT, T2D obese subjects. Nevertheless, *ANKRD26* mRNAs tended to be lower in the IGT and T2D obese compared with the NGT obese individuals, indicating that the *ANKRD26* gene expression not only is negatively related in humans to increased body mass but it might be further down-regulated by the fluctuations of glucose levels, which are common in glucose intolerant and in diabetic subjects (Figure 10). However, no conclusive statement may be assumed from these data and further studies in more numerous obese sub-phenotype groups will be required to address this last question.

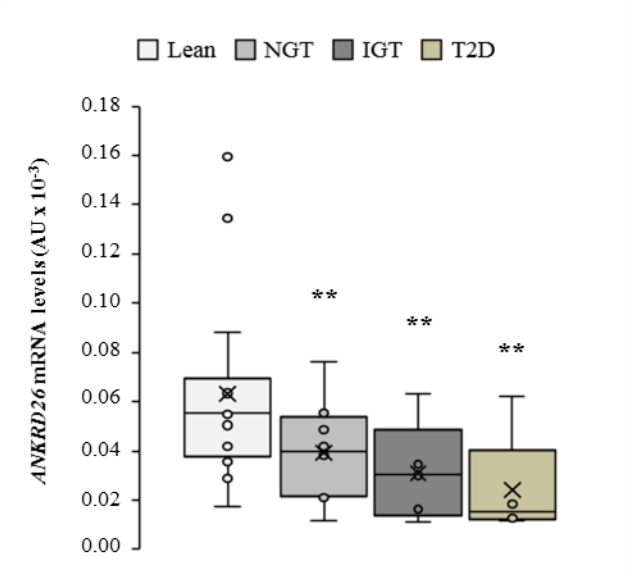

Figure 10. *ANKRD26* mRNA levels in PBLs of lean and obese sub-phenotypes. *ANKRD26* mRNA levels by qPCR in 14 lean controls and 10 NGT, 5 IGT and 5 T2D obese subjects. Values are mean ± SD. Significances (by t-test) were calculated between lean and obese group. ***p*<0.01 Vs. lean group. **NGT:** Normal glucose tolerant, **IGT:** Impaired glucose tolerant, **T2D:** Type 2 Diabetic

In the meantime, I began to investigate also the question whether the DNA methylation at the *ANKRD26* promoter occurs even in humans and is related to changes in the body mass. I thus addressed this point by measuring the DNA methylation status of the whole *ANKRD26* promoter using a bisulfite sequencing approach in the PBLs genomic DNA from 6 subjects, 3 featuring the lowest BMI (lean) and an equal number of subjects exhibiting the largest BMI (obese). For the analysis, the whole *ANKRD26* promoter was arbitrarily divided in 5 CpGs rich sub-regions, which also include the 5' Untranslated Region (5'-UTR) and the first exon, as follows: sub-region S1, -991/-693 bp; S2, -716/-370 bp; S3, -349/-48 bp; S4, -68/+157 bp and S5, +134/+390 bp (Figure 11).

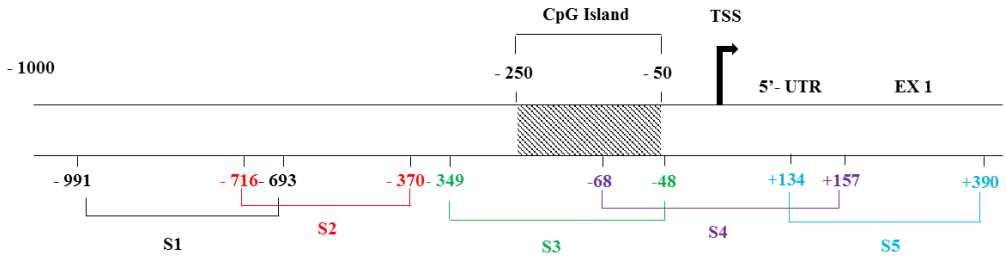

Figure 11. Schematic representation of the *ANKRD26* promoter. Sub-region S1: -991/-693 bp; S2: -716/-370 bp; S3: -349/-48 bp; S4: -68/+157 bp and S5: +134/+390 bp. CpG island: -250/-50 bp. **TSS:** Transcription Start Site, **5'UTR:** 5' Untraslated Region, **Ex 1:** first exon.

In the 3 lean subjects, the bisulfite sequencing analysis of the *ANKRD26* promoter revealed that a high density of CpGs methylation at the sub-region S1 (~85%), a low/moderate CpGs methylation at the sub-rigion S2 (~20%) and no CpGs methylation at the sub-regions S3, S4 and S5 (Table 9). Interestingly, in the 3 obese individuals, the same analysis showed almost similar CpGs methylation pattern at the sub-region S1, which results hyper-methylated, and at the sub-regions S3, S4 and S5, which are characterized by a global DNA hypo-methylation. Differently, the sequencing analysis revealed a massively increased methylation levels at the sub-region S2 (~35%) in these latter individuals.

Position	***% of DNA Methylation***	
	Lean	**Obese**
S1; *-991/-693*	85.9±2.1	85.2±3.6
S2; *-716/-370*	21.1±9.7	33.7±10.0
S3; *-349/-48*	3.7±4.6	1.0±0.9
S4; *-68/+157*	0.3±0.5	0.5±0.1
S5; *+134/+390*	0.2±0.1	0.4±0.2

Table 9. DNA methylation enrichment in the *ANKRD26* promoter. Percentage of DNA methylation of the 5 sub-regions analyzed in lean and obese subjects. **S1**: sub-region 1, **S2:** sub-region 2, **S3:** sub-region 3, **S4:** sub-region 4 and **S5:** sub-region 5. Values are mean ± SD.

Indeed, in the 3 subjects with the higher BMI compared with 3 individuals with the lower BMI, the sub-region S2 featured a higher density of DNA methylation in 3 close CpG dinucleotides at -689 bp, -659 bp and -651 bp from the *ANKRD26* TSS (Figure 12a). In addition, plotting the combined percentage of the DNA methylation at these 3 cytosine residues of the 6 selected subjects in relation with their *ANKRD26* gene expression revealed that this specific DNA methylation at the *ANKRD26* promoter inversely correlates with the amounts of the *ANKRD26* mRNA levels in humans (n=6, r=-0.850, $p<0.05$) (Figure 12b). These data indicate that in the obese individuals the *ANKRD26* mRNA down-regulation is accompanied by a specific increase of the *ANKRD26* promoter DNA methylation status in a specific restricted region and confirm my hypothesis that fat mass increase may modulate the *ANKRD26* gene expression through epigenetic changes in humans. DNA methylation often induces gene silencing by inhibiting transcriptional activator binding to promoters [109]. Interestingly, a bioinformatic analysis for the identification of transcription factor binding sites on the *ANKRD26* promoter region, predicted also in humans a consensus sequence within the sub-region S2 (-702 bp/-687 bp) for the histone acetyltransferase/transcriptional coactivator p300, hypothetically suggesting that hyper-methylation at this region could interfere with p300 binding to the *ANKRD26* promoter and in turn may contribute to the *ANKRD26* down-regulation. However, whether p300 is also involved in the epigenetic regulation of the *ANKRD26* gene and whether a direct causal relationship between the promoter DNA methylation and the transcription of the *ANKRD26* gene exists are not yet known and are currently under investigation.

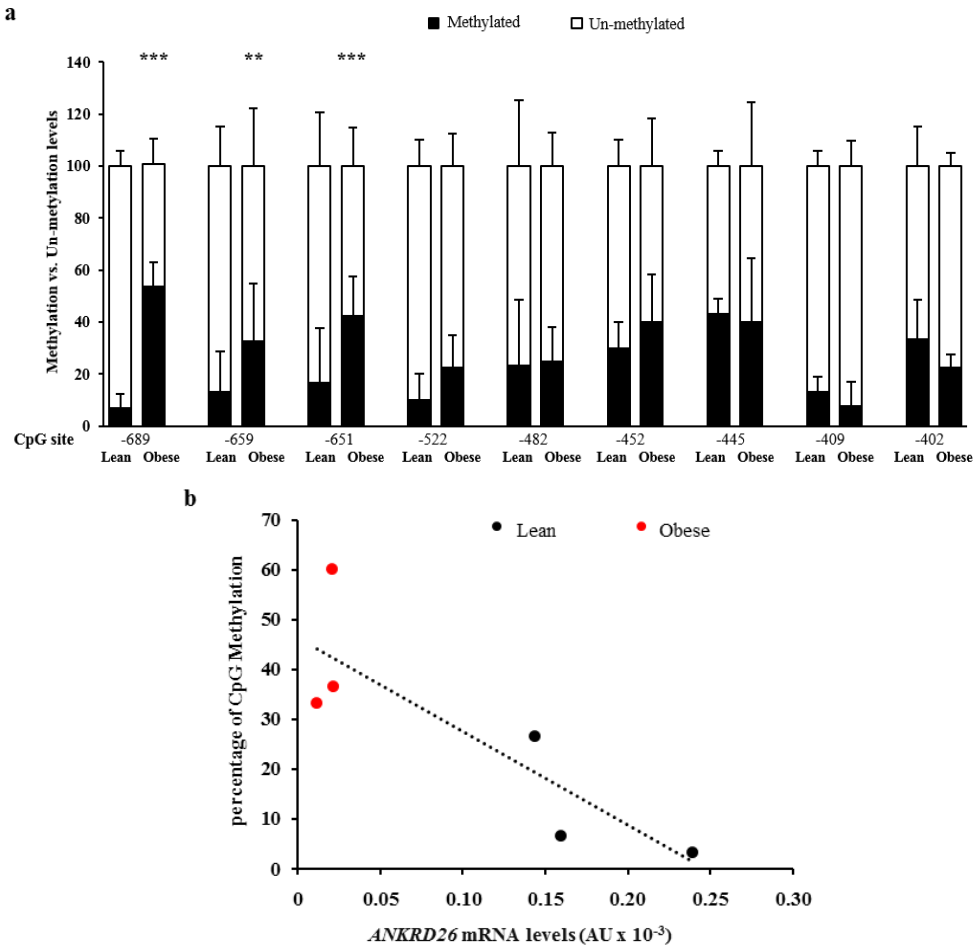

Figure 12. DNA methylation status at the sub-region S2. a) Methylation analysis of the whole S2, which includes 9 CpG sites: -689, -659, -651, -522, -482, -452, -445, -409, -402 bp from the TSS of the *ANKRD26* gene. Values are mean ± SD. Significances (by t-test) were calculated between lean and obese group. **p<0.01, ***p<0.001 **b)** Correlation analysis of the CpGs -689, -659, -651 in relation to the *ANKRD26* mRNA levels. Values are the sum of the percentage of the 3 CpG sites in lean and obese subjects Vs. the *ANKRD26* mRNA levels (n=6, r=-0.850, p<0.05). Red circles are related to lean subjects, black circles are related to obese subjects.

In conclusion, this study allowed me to demonstrate that the *ANKRD26* gene is sensitive to epigenetic regulation even in humans and that the down-regulation of the *ANKRD26* gene expression and the site specific CpG hyper-methylation of its promoter represent a common abnormality in obese patients. Furthermore, my study allowed me also to demonstrate that PBLs are a valid non-invasive proxy system for the evaluation of changes of gene expression and DNA methylation occurring on *ANKRD26* gene in humans. However, whether the epigenetic down-regulation of the *ANKRD26* gene precedes or is subsequent to obesity and thus whether the evaluation of the *ANKRD26* promoter methylation status in blood samples may be used for prediction of obesity onset are still opened question that in the future I will address with *ad hoc* investigations.

Altered expression of the miRNAs hsa-miRNA-23a-5p, -193a-5p, and -193b-5p precedes adipocyte hypertrophy in First Degree Relatives of T2D patients

Concerning the second study, as I previously reported, a family history of diabetes is associated with a range of metabolic abnormalities and is a strong risk factor for the development of T2D [16]. Indeed, first degree relatives of T2D patients (T2D-FDRs) have up to 10-fold higher risk of developing the disease than age- and weight-matched subjects without a clear family history of the disease [17, 18], and frequently have an impaired nonoxidative glucose metabolism long before the onset of T2D, and decreases in insulin and amylin release in response to glucose stimulation [18]. Furthermore, similarly to obese individuals, T2D-FDRs show larger scAT cells characterized by inappropriate hypertrophy due to an impaired ability to recruit and/or differentiate new adipose cells to store excessive lipids [21]. The mechanisms driving the restricted adipogenesis occurring in T2D-FDRs are currently unclear but may depend on epigenetic changes rather than genetic origins.

In this context, I investigated by an Epigenome Wide analysis whether changes in the miRNAs expression occurs in individuals with familiarity for T2D and whether those changes relate with hypertrophy of subcutaneous mature adipocyte size.

To address these questions, I selected and analyzed a group of 20 well-characterized lean euglycemic subjects, 9 of them with a known family history of T2D and 11 exhibiting no familiarity for the disease. While there were no significant differences in age, BMI and body fat percentage (%) among the 2 groups, the T2D-FDRs had elevated fasting blood glucose ($p<0.05$) and fasting insulin ($p<0.001$) levels compared with the individuals lacking familiarity for T2D. Furthermore, the OGTT and the euglycemic clamp tests (GIR/bw) revealed a reduced glucose tolerance ($p<0.05$) and insulin sensitivity ($p<0.01$), respectively, in individuals with family history of T2D. It is noteworthy that the T2D-FDRs had larger subcutaneous fat cells ($p<0.001$) when compared to the control subjects, remarking that an inappropriate enlargement of the abdominal adipose cells is a common feature in subjects with family history of T2D. The clinical characteristics of the two analyzed cohorts are detailed in Table 10.

Then, in order to evaluate the miRNAs profile and investigate the epigenetic mechanisms driving the restricted adipogenesis associated to familiarity of T2D, I evaluated by a NGS approach (miRNAs-seq) the miRNAome of the stromal-vascular fraction cells (SVFs), obtained from the scAT biopsies of both the healthy T2D-FDRs and the individuals with no family history of T2D. The human SVFs are indeed a specific sub-population of cells of the adipose tissue, which are rich in pre-adipocytes, and are commonly used to evaluate adipocyte commitment and/or differentiation *in vitro* [110]. This makes these cells the ideal cellular model for this study.

Measure	*CTRLs*	*T2D-FDRs*
N	11	9
Age, years	39.4 ± 7.8	42.3 ± 8.7
BMI, Kg/m2	24.5 ± 2.2	25.4 ± 1.5
Fat percent, %	24.3 ± 6.3	26.9 ± 7.3
Cell size, µm	89.6 ± 6.0	100.2 ± 5.2***
GIR/bw, mg/min	11.3 ± 2.5	7.9 ± 1.7**
f-insulin, µU/mL	34.0 ± 13.3	60.1 ± 22.7**
fb-glucose, mmol/L	4.4 ± 0.4	4.8 ± 0.4*
OGTT p-glucose 2h, mmol/L	4.9 ± 1.2	6.6 ± 1.7*

Table 10. Characteristics of the analyzed population. BMI: body mass index, **GIR/bw:** glucose infusion rate/body weight, **fb:** fasting blood, **OGTT:** oral glucose tolerance test. Values are mean ± SD. Significances (by t-test) were calculated between the two groups. *p<0.05, **p<0.01, ***p<0.001.

The analysis of the miRNAs profile in the SVFs pooled total RNA preparations from the lean euglycemic subjects with a known family history of T2D and of their counterpart exhibiting no familiarity for the disease revealed that 34 miRNAs were found to be differentially expressed (false discovery rate-adjusted *p*-value cut-off of 0.05) among the two groups, of which 23 were down-regulated and 11 were up-regulated in the T2D-FDRs compared with the control individuals. Then, to confirm these computational data, I proceeded my profiling analysis by validating the expression of 11 out of 34 miRNAs by qPCR assay in 18 subjects of the selected population for individual testing. The 11 miRNAs were selected applying the arbitrary chosen cut-off of a 50% increased or decreased expression in the T2D-FDRs compared with the control subjects. From this analysis resulted that the expression of 3 miRNAs, the *hsa-miR-23a-5p*, the *hsa-miR-193a-5p* and the *hsa-miR-193b-5p*, were down-regulated in the T2D-FDRs and was in line with the data obtained by the sequencing. In particular, the *hsa-miR-23a-5p* was decreased of about 40% (Ctrl group: 0.0055 ± 0.0013 AU; T2D-FDR group: 0.0035 ± 0.0012 AU; p<0.01), the *hsa-miR-193a-5p* resulted to be decreased of about 30% (Ctrl group: 0.0415 ± 0.0129 AU; T2D-FDR group: 0.0295 ± 0.0100 AU; p<0.05), and the *hsa-miR-193b-5p* was down-expressed of about 25% (Ctrl group: 0.0386 ± 0.0082 AU; T2D-FDR group: 0.0282 ± 0.0083 AU; p<0.01) in the T2D-FDR group compared with the controls (Figure 13). These data suggest that specific changes in the miRNAs expression profile occur in individuals with familiarity for T2D.

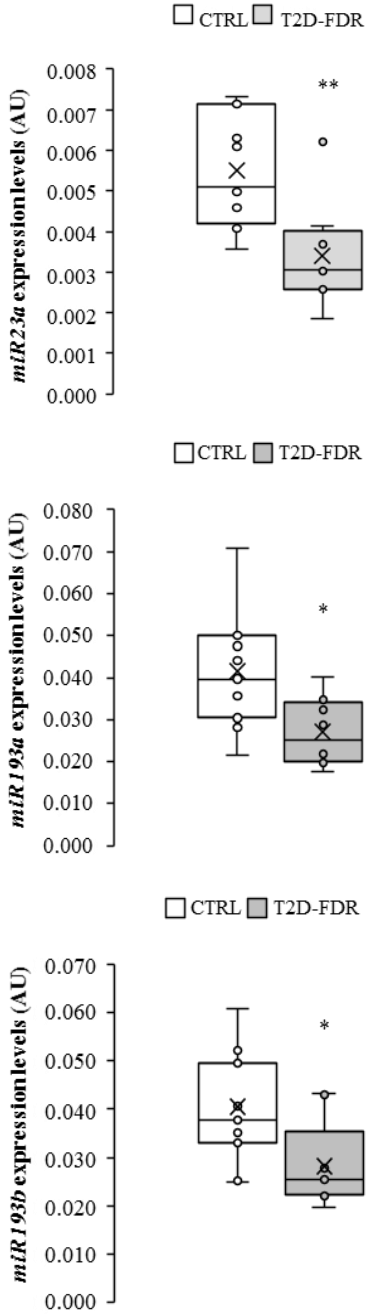

Figure 13. Validation of miRNA-seq data. The *hsa-miR23a-5p*, *hsa-miR193a-5p* and *hsa-miR193b-5p* levels by qPCR in 8 T2D-FDRs and 10 subjects without family history of T2D. Values are mean ± SD. Significances (by t-test) were calculated between the two groups. **p*<0.05, ***p*<0.01.

Furthermore, to investigate the biological relevance of these 3 miRNAs, I explored the significance of the altered expression of the *hsa-miR-23a-5p*, *hsa-miR-193a-5p* and *hsa-miR-193b-5p* in relation to human adipose tissue development and function. I thus plotted the expression of each identified

miRNA in the 18 selected subjects in relation with their subcutaneous mature adipocyte size to have an indirect esteem of the effect of each identified miRNA on adipocyte precursor cell commitment/differentiation. Indeed, as I previously reported in this thesis, restricted adipogenesis in human subcutaneous adipose tissue is determined by impaired adipocyte precursor cell commitment and results in hypertrophy of adipocytes which are resident in the adipose tissue [26]. Furthermore, recent reports revealed that these abnormalities predict T2D independently of obesity [75]. Notably, the *hsa-miR-23a-5p* expression in the preadipocytes exhibited a significant negative correlation with the size of the mature subcutaneous adipose cells from these same individuals (Figure 14a; n=18, r=-0.4998, $p<0.05$). Similarly, significant negative correlation were between subcutaneous adipocyte size and *hsa-miR-193a-5p* expression (Figure 14b; n=18, r=-0.6491, $p<0.01$), and between subcutaneous adipocyte size and *hsa-miR-193b-5p* expression (Figure 14c; n=18, r=-0.4826, $p<0.05$). These data indicate that low expression of the *hsa-miR-23a-5p, hsa-miR-193a-5p* and *hsa-miR-193b-5p* not only is a common feature in T2D-FDRs, but also precedes development of excessive subcutaneous adipocyte size in subjects with familiarity of T2D and might predict hypertrophy.

Then, based on the last results, I investigated whether the selected 3 miRNAs may potentially modulate gene pathways in such manner involved in adipose tissue development and function (Figure 15). To this aim, by using 4 well-known tools for miRNA target prediction (miRanda, Targetscan, RNA22 and miRWalk) I identified the combined target mRNAs for each selected miRNA and I questioned each list of combined target mRNAs for pathway prediction by a Protein ANnotation Through Evolutionary Relationship (PANTHER) classification analysis. In accordance with the PANTHER classification system ($p<0.05$), for the *hsa-miR-23a-5p* I identified, among several pathways, as the most enriched the WNT signaling pathway, which plays a key role in the regulation of the adipocyte commitment and differentiation, and the inflammation mediated by chemokine and cytokine signaling pathway, which includes gene regulating adipocyte secreting properties (Figure 15a). Interestingly, the same analysis made for the combined target mRNAs of the *hsa-miR-193a-5p* and *hsa-miR-193b-5p* revealed the WNT signaling pathway and the inflammation mediated by chemokine and cytokine signaling pathway as the most enriched (Figure 15b and c). Altogether, these data suggest that the changes in the miRNAs expression profile which occurs in the individuals with familiarity for T2D may represent a mechanism responsible for the impaired adipose cell recruitment by interfering with functions of adipocyte specific pathways.

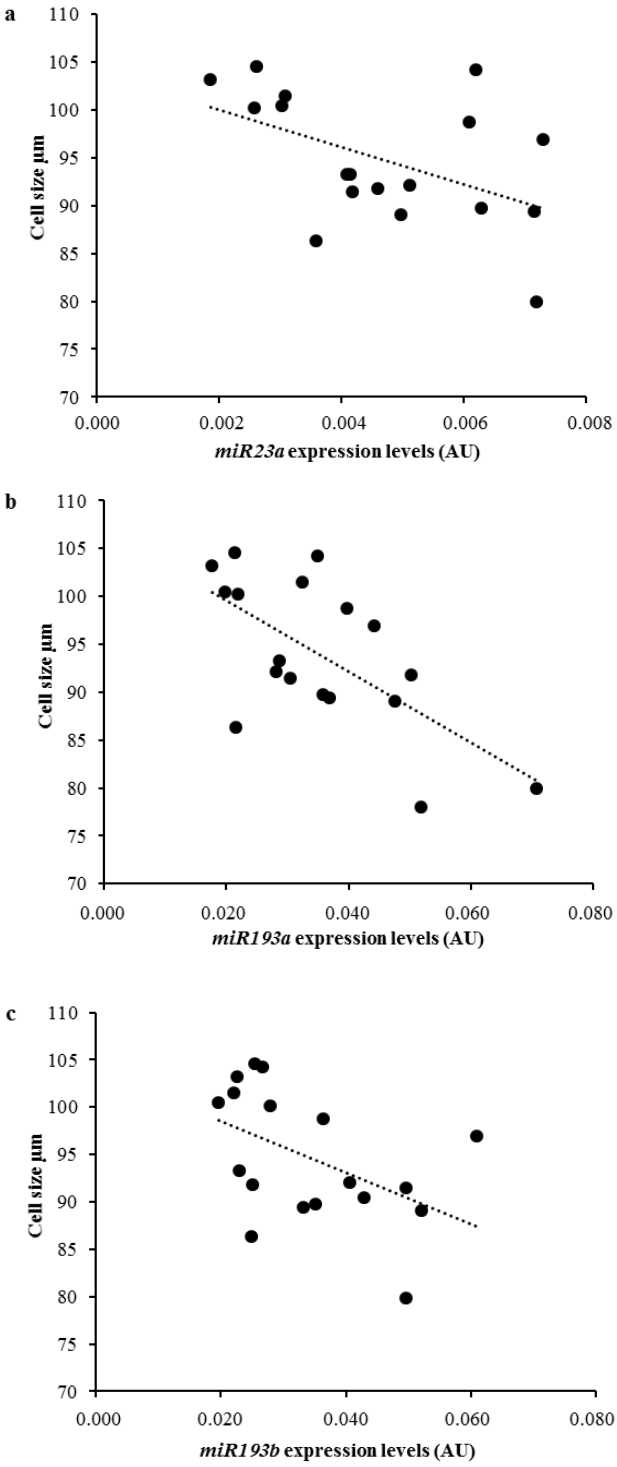

Figure 14. Correlation analysis between miRNAs expression and adipocyte cell size. a) *hsa-miR-23a-5p* Vs. adipocyte cell size (n=18, r=-0.4998, $p<0.05$). **b)** *hsa-miR-193a-5p* Vs. adipocyte cell size (n=18, r=-0.6491, $p<0.01$). **c)** *hsa-miR-193b-5p* and adipocyte cell size (n=18, r=-0.4826, $p<0.05$).

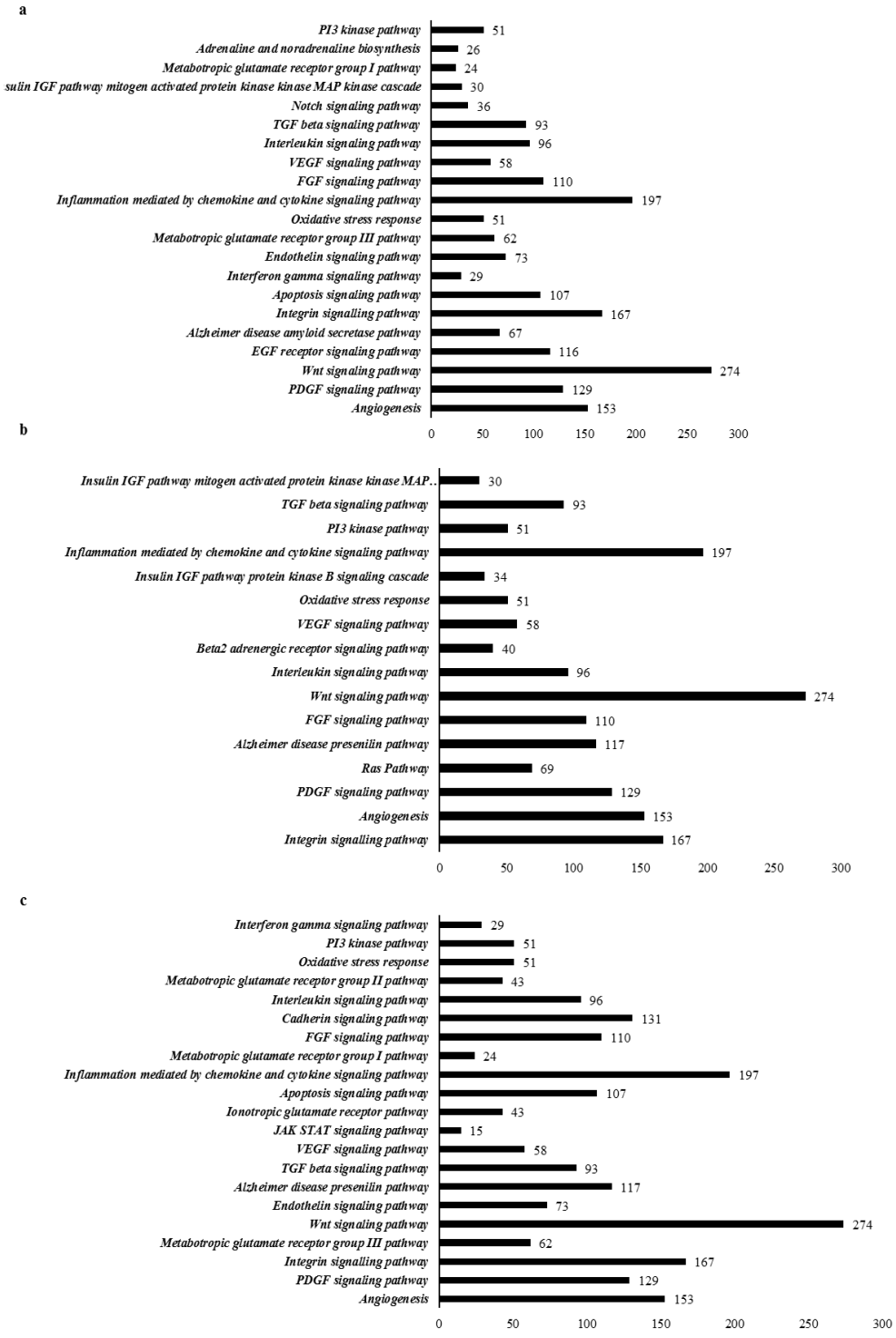

Figure 15. PANTHER analysis on the combined target mRNAs of the 3 selected miRNAs. **a)** PANTHER analysis of the mRNA target of the *hsa-miR-23a-5p*. **b)** PANTHER analysis of the mRNA target of the *hsa-miR-193a-5p*. **c)** PANTHER analysis of the mRNA target of the *hsa-miR-193b-5p*.

Lastly, I searched for association between the specific epigenetic marks previously identified with the miRNA-seq and specific functional consequences on gene expression investigating the genome-wide expression changes associated with familiarity for T2D by RNA-seq. The analysis of the transcriptome in the SVFs pooled total RNA preparations from the lean euglycemic subjects with a known family history of T2D and of their counterpart exhibiting no familiarity for the disease revealed that 84 gene were found to be differentially expressed (DEGs) among the two groups (false discovery rate-adjusted *p*-value cut-off of 0.05), of which 24 were down-regulated and 60 were up-regulated in the T2D-FDRs compared with the control individuals. Furthermore, to investigate the biological relevance of the identified DEGs, I performed a PANTHER classification analysis. Notably, according with the PANTHER classification system ($p<0.05$), as in the case of the previous analysis on the miRNA-target genes I identified among several pathways closely related to metabolic and differentiation processes the WNT signaling pathway and the inflammation mediated by chemokine and cytokine signaling pathways (Figure 16). Altogether, these computational data suggest that changes in the gene expression profile also occurs in individuals with familiarity for T2D and that these alterations of gene expression may in part account for the impaired adipose cell recruitment typical of T2D-FDRs.

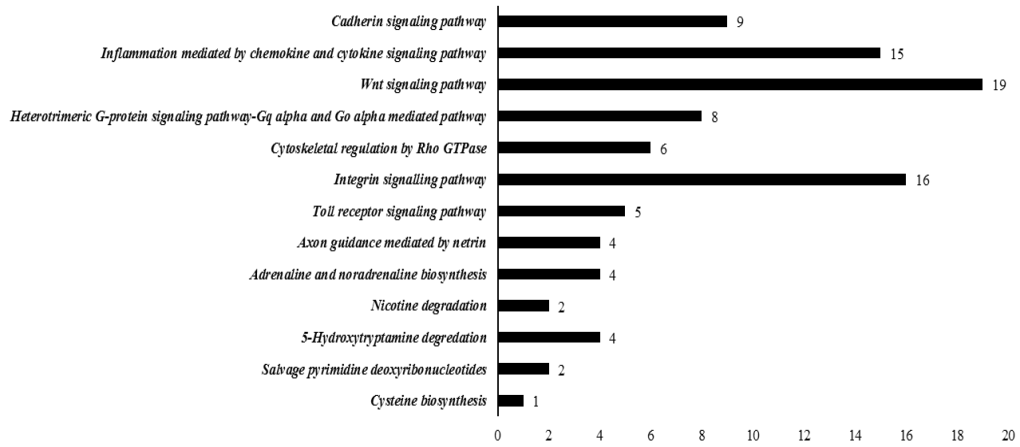

Figure 16. PANTHER analysis on the 84 DEGs.

Then, I proceeded my profiling analysis by validating the expression of 8 DEGs of out 84, which resulted using the miRWalk software to be target of at least 1 of the previous 3 identified miRNAs, in 18 subjects of the selected population for individual testing. From this analysis resulted that the expression of the *PTPRD*, *IGF2*, *INMT*, *MXRA5*, *RGS4*, *SYNPOL2L* and *PRELP* genes, but not *OXTR* gene, was up-regulated in healthy T2D-FDRs compared with the individuals with no family history of T2D (Figure 17), confirming thus the hanging trends of gene expression from the RNA-Seq analysis.

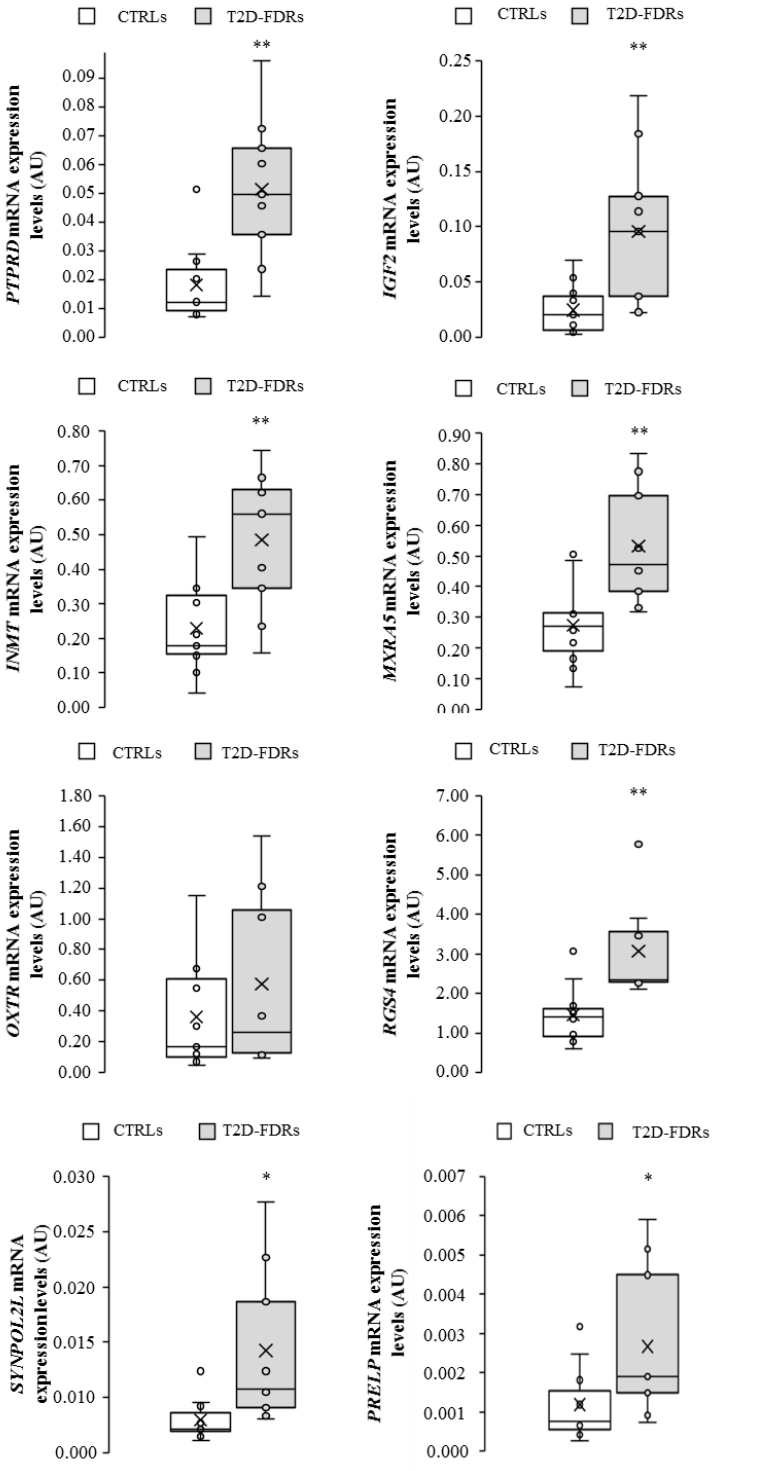

Figure 17. Validation of RNA-seq data. The mRNA expression of the 8 DEGs levels was determined by qPCR in 8 T2D-FDRs and 10 subjects without family history of T2D. Values are mean ± SD. Significances (by t-test) were calculated between the two groups. *p<0.05, **p<0.01. **DEGs:** Differentially expressed genes.

In particular, in the subjects with familiarity of T2D, *PTPRD* gene expression was increased of about 2.7 fold (Ctrl group: 0.0192 ± 0.0133 AU; T2D-FDR group: 0.0525 ± 0.0253 AU; $p<0.01$); *IGF2* gene resulted to be increased of about 3.8 fold (Ctrl group: 0.0246 ± 0.0222 AU; T2D-FDR group: 0.0959 ± 0.0718 AU; $p<0.01$); *INMT* gene expression was increased of about 2.3 fold (Ctrl group: 0.02158 ± 0.0149 AU; T2D-FDR group: 0.0494 ± 0.0235 AU; $p<0.01$); *MXRA5* mRNA levels were increased of about 1.8 fold (Ctrl group: 0.2814 ± 0.1470 AU; T2D-FDR group: 0.5140 ± 0.1729 AU; $p<0.01$); *RGS4* gene was up-regulated of about 1.9 fold (Ctrl group: 1.459 ± 0.731 AU; T2D-FDR group: 3.067 ± 1.277 AU; $p<0.01$); *SYNPOL2L* gene expression was increased of about 3.1 fold (Ctrl group: 0.0029 ± 0.0018 AU; T2D-FDR group: 0.0092 ± 0.0070 AU; $p<0.05$); *PRELP* gene expression was increased of about 2.2 fold (Ctrl group: 0.0011 ± 0.0009 AU; T2D-FDR group: 0.0026 ± 0.0019 AU; $p<0.05$) No statistical differences were observed among the 2 groups for *OXTR* gene expression (Ctrl group: 0.3604 ± 0.3578 AU; T2D-FDR group: 0.5759 ± 0.5842 AU) (Figure 17).

Furthermore, to define the association between the miRNAs, *hsa-miR-23a-5p*, *hsa-miR-193a-5p* and *hsa-miR-193b-5p*, and their target genes, I performed a correlation analysis between the expression levels of each miRNA and its target gene (Table 11). This analysis revealed that the *hsa-miR-23a-5p* expression inversely correlates with the mRNA levels of *IGF2*, *MXRA5*, *OXTR* and *RGS4* genes; while both the *hsa-miR-193a-5p* and *hsa-miR-193b-5p* expressions inversely correlate with the mRNA levels of *IGF2* gene. These data suggest that the 3 identified miRNAs not only may effectively bind their target genes, but in the context of my study the low expression of the *hsa-miR-23a-5p*, *hsa-miR-193a-5p* and *hsa-miR-193b-5p* represent a common feature in T2D-FDRs, that may predispose to adipocyte hypertrophy by modulating in the pre-adipocytes the expression of specific genes such as *IGF2* and *MXRA5*.

	hsa-miR-23a-5p		
Genes	**n**	**r**	***p value***
IGF2	18	-0.588	*<0.01*
MXRA5	18	-0.696	*<0.001*
OXTR	18	-0.454	*<0.05*
RGS4	18	-0.465	*<0.05*
PRELP	18	0.061	*n.s.*

	hsa-miR-193a-5p		
Genes	**n**	**r**	***p value***
IGF2	18	-0.556	*<0.01*
INMT	18	-0.425	*n.s.*
OXTR	18	-0.376	*n.s.*
RGS4	18	-0.040	*n.s.*
SYNPOL2L	18	-0.232	*n.s.*
PRELP	18	0.188	*n.s.*

	hsa-miR-193b-5p		
Genes	**n**	**r**	***p value***
IGF2	18	-0.515	*<0.05*
PTPRD	18	-0.287	*n.s*
INMT	18	-0.408	*n.s*
SYNPOL2L	18	-0.314	*n.s*
PRELP	18	0.129	*n.s*

Table 11. Correlation analysis between miRNAs and mRNA target genes. *hsa-miR-23a-5p, -193a-5p,* and *-193b-5p* expression Vs. target gene expression (n=18).

In support of the last hypothesis, the expression levels of both the *IGF2* (n=18, r=0.4718, $p<0.05$) and the *MXRA5* genes (n=18, r=0.5848, $p<0.01$) positively correlate with adipocyte cell size (Figure 18a and b). Notably, elevated concentrations of the Insulin like Growth Factor 2 (IGF2) prevent terminal adipocyte differentiation in hemangioma stem cells *in vitro* [111], and, interestingly, increasing levels of serum IGF2 occur in obese individuals compared with lean subjects, and the hormone concentrations are even higher in subjects which present both obesity and T2D [112-113]. Overall, the combinations of the last observations with my findings showing increased *IGF2* gene expression in pre-adipocytes from individuals with family history of T2D suggest that familiarity for T2D, as well as obesity and T2D itself, presents in the up-regulation of *IGF2* a common feature which may cause impaired ability to recruit and/or differentiate new adipose cells.
Regarding the *Matrix Remodeling Associated 5* (*MXRA5*) gene, recent studies instead revealed that the *MXRA5* gene is predominantly expressed in human pre-adipocytes compared with its expression in human mature adipocytes [114], and furthermore, that the *MXRA5* gene was in a list of 15 top ranked down-regulated genes in scAT biopsies from obese subjects upon weight loss and weight maintenance [115]. Therefore, even though not much is known about the function of this gene, these observations may suggest a potential role

of *MXRA5* in the extracellular matrix regulation in pre-adipocytes, and my findings may sustain a potential role of *MXRA5* gene in the impaired recruitment and/or differentiation of new adipose cells in T2D-FDRs. However, whether in T2D-FDRs the altered expression of the miRNAs, *hsa-miR-23a-5p*, *-193a-5p* and *-193b-5p*, may actually predispose to adipocyte hypertrophy by modulating in the pre-adipocytes the expression of *IGF2* and *MXRA5* is not yet known and will be object of further investigations.

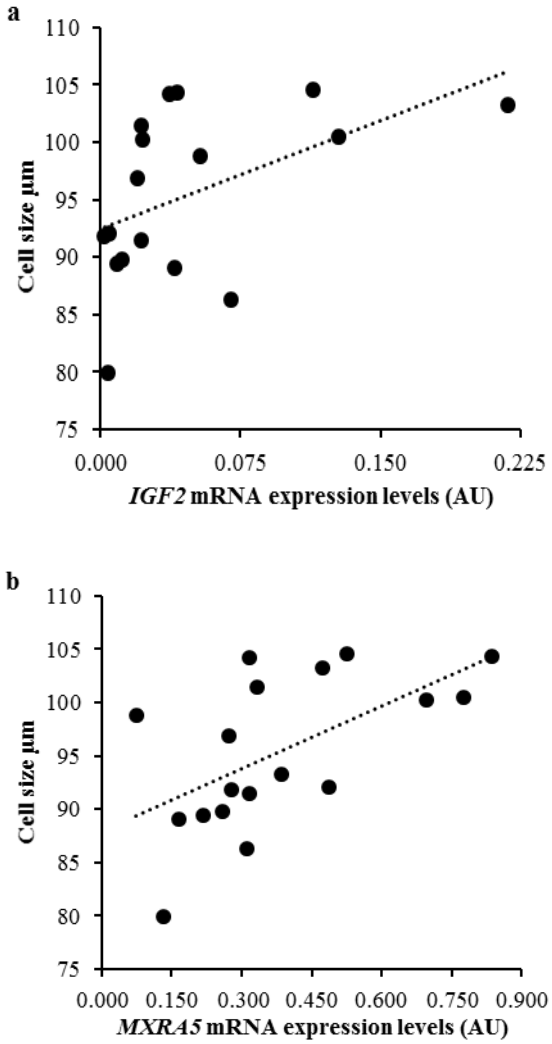

Figure 18. Correlation analysis between *IGF2* and *MXRA5* mRNA expression and adipocytes cell size. a) *IGF2* mRNA expression levels Vs. adipocyte cell size (n=18, r=0.4718, $p<0.05$). **b)** *MXRA5* mRNA expression levels Vs. adipocyte cell size (n=18, r=0.5848, $p<0.01$).

In conclusion, this second study allowed me to demonstrate that the specific down-regulation of the miRNAs, *hsa-miR-23a-5p*, *-193a-5p* and *-193b-5p*, is a common feature in individuals with familiarity of T2D, and inversely correlate to adipocyte hypertrophy in humans. Furthermore, my data suggest that the altered expression of the miRNAs, *hsa-miR-23a-5p*, *-193a-5p* and *-193b-5p*, may actually predispose to adipocyte hypertrophy by impairing pre-adipocytes commitment and/or differentiation through the modulation in these cells of the mRNA expression of specific target genes, such as *IGF2* and *MXRA5*. Follow-up studies of T2D-FDR individuals are necessary to further elucidate the effect of *hsa-miR-23a-5p, -193a-5p,* and *-193b-5p* levels in the progression to T2D. Moreover, future investigations, extended in T2 diabetics and in other subjects with high risk of T2D, will give the opportunity to figure out whether the evaluation of the expression of these 3 miRNAs will be a reliable system for prediction of T2D and will offer new chance for the development of novel pharmacological strategies for the treatment of diabetes.

Conclusions and future perspectives

The epigenome undergoes continuous transformations throughout our own lifetime leading to changes in genome function. The epigenetic hypothesis argues that, in addition to genetic variation, epigenetics provides an additional set of mechanisms mediating the relationship between genotype and the environment, contributing to the individual susceptibility to different disorders. During the past decades, the study of epigenetic modifications has been one of the most emerging and novel areas in fundamental as well as in clinical research and currently represents a very productive field of study, which has already provided a framework for the search of etiological factors in environment-associated diseases such as T2D and obesity. In my PhD thesis, I dealt the issue of the epigenetic profiling with two distinct and separate approaches in populations at high risk of T2D.

From the first part of my experiments (candidate gene analysis), I can conclude that the *ANKRD26* gene expression inversely correlate with BMI and its down-regulation represents a common abnormality in obese patients. Furthermore, in these individuals, the *ANKRD26* mRNA levels are strongly negatively correlated with the DNA methylation status of a specific region of the *ANKRD26* promoter, suggesting that this gene is sensitive to epigenetic regulation in human obesity. Whether the epigenetic down-regulation of the *ANKRD26* gene precedes or is subsequent to obesity and thus whether the increased CpG methylation of the *ANKRD26* promoter may be predictive of obesity onset is not yet known and deserves further and ad hoc investigation.

From the second part of experiments (EWAS), I can conclude that the down-regulation of *the hsa-miR-23a-5p, -193a-5p,* and *-193b-5p* is common in First Degree Relatives of T2D patients and their expression levels inversely correlate with subcutaneous adipocyte cell size. Furthermore, the bioinformatic analysis of the miRNA-target mRNA highlighted specific enrichments for pathways associated to adipocyte commitment and differentiation. I might thus hypothesize that in T2 diabetic FDRs changes of the *hsa-miR-23a-5p, -193a-5p*, and *-193b-5p* expression not only precede adipocyte hypertrophy, but it might be responsible for restricted adipogenesis in these individuals. Thus, follow-up studies of T2D-FDR individuals are necessary to further elucidate the effect of *hsa-miR-23a-5p, -193a-5p*, and *-193b-5p* levels in the progression to T2D.

However, even though we are still far from the understanding of the epigenetics of this complex disease, the work made by us and by other groups will pave the way to novel and more effective strategies aimed at diabetes prevention and at personalized epigenetic treatment. Indeed, it becomes clear that getting a full picture of the epigenetic events involved in this disease will (1) represent a powerful tool for predicting and preventing future disease onset

in the population; (2) provide additional stimuli for the development of clinical epigenetic biomarkers, which will generate novel relevant information for diagnosis, prognosis and therapy optimization; and (3) drive advancement in epigenetic drug discovery with the generation of more effective epi-drugs, selective for specific epi-targets, which in the forthcoming future might be used in combination with conventional therapeutics and/or might get the opportunity to develop epigenetic treatment personalized to the patient's epigenetic traits.

Acknowledgements

Ebbene si, la parte più difficile da scrivere è proprio questa, difficile in quanto esprimere in poche righe la gratitudine verso coloro che hanno contribuito alla nascita e allo sviluppo di questa tesi non è facile, le parole non sono mai state il mio punto di forza, ma ci proverò nelle righe seguenti.

Per prima cosa vorrei ringraziare il Prof. Francesco Beguinot non solo per avermi accolto presso i suoi laboratori e per avermi dato l'opportunità di crescere dal punto di vista professionale, ma per essere stato per me una guida, un esempio da seguire per la passione e la dedizione che ha per la ricerca scientifica, per avermi accolto all'interno di una grande famiglia della quale ogni singolo componente nel suo piccolo ha contribuito nel corso di questi anni alla mia crescita personale e professionale.

Ringrazio il coordinatore del programma di dottorato, il Prof. Gianni Marone per l'assidua a competente supervisione scientifica.

Ringrazio la Dott.ssa Claudia Miele per i consigli, per essermi stata sempre accanto in questo percorso, per aver creduto in me, per i momenti condivisi, per essersi presa cura di me in questi anni e per avermi incoraggiato a dare sempre il massimo.

Ringrazio il Prof. Pietro Formisano per avermi incoraggiato ad intraprendere questo percorso, per aver creduto in me, per la sua disponibilità e cortesia, e per il sostegno in questi anni.

Un ringraziamento particolare va al Dott. Gregory Alexander Raciti per i preziosi insegnamenti, per le ore dedicate alla mia tesi, per il supporto mostratomi durante i momenti di difficoltà, per aver creduto in me quando io stessa non ci credevo e per aver contribuito a rendermi la persona che sono oggi.

Vorrei ringraziare la Dott.ssa Rosa Spinelli non solo compagna di banco e di scrivania ma amica fidata. Ringrazio Rosa per i suoi insegnamenti, per gli incoraggiamenti, per il sostegno nei momenti difficili, che non sono mancati, per aver gioito insieme nei momenti felici, per aver condiviso tanto in questi anni, è stata ed è tuttora per me una vera amica più che una collega.

Ringrazio il Dott. Michele Longo ed il Dott. Massimiliano Volpe per essermi stati vicini ancor prima che intraprendessi questo percorso, per aver sempre creduto in me, per l'aiuto in questi anni, per avermi spronato a dare sempre il massimo e per avermi aiutato a capire che questo era ciò che davvero volevo fare nella vita. Forse senza il loro supporto non avrei mai intrapreso questo percorso, grazie di cuore davvero.

Ringrazio i miei amici e colleghi di laboratorio in particolare la Dott.ssa Paola Mirra, il Dott. Luca Parrillo, la Dott.ssa Cecilia Nigro, la Dott.ssa Federica Zatterale, la Dott.ssa Immacolata Prevenzano ed il Dott. Michele Campitelli non solo per l'aiuto con gli esperimenti, ma per aver contribuito a rendere questo difficile percorso un pezzo di vita che ricorderò sempre con gioia e affetto, ed i miei colleghi del programma di dottorato la Dott.ssa Alessia Leone, il Dott. Domenico Conza e la Dott.ssa Serena Ricci, condividere con voi questa esperienza è stato fantastico, mi ha aiutato a non sentirmi mai sola e ad affrontare a testa alta tutte le difficoltà, grazie ragazzi.

Ringrazio Annalisa Pollio per l'aiuto con gli esperimenti e per il tempo trascorso insieme, spero di essere riuscita a trasmette a lei l'amore che ho per la ricerca così come i miei tutor hanno fatto con me.

Vorrei ringraziare i miei genitori i miei nonni, mio fratello e tutti i miei amici (la mia famiglia) per il loro incrollabile sostegno morale, per avermi aiutato nel raggiungimento di questo nuovo traguardo e per avermi insegnato che non bisogna arrendersi mai.

Un ringraziamento speciale ad Angelo per la sua incrollabile pazienza, per essermi rimasto sempre accanto e per credere in me più di quanto io stessa sia capace.

Grazie di cuore a tutti…questo pezzo di vita lo porterò sempre con me!

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International Journal of Obesity (2016) **40,** 929–937

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ORIGINAL ARTICLE

Hoxa5 undergoes dynamic DNA methylation and transcriptional repression in the adipose tissue of mice exposed to high-fat diet

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BACKGROUND/OBJECTIVES: The genomic bases of the adipose tissue abnormalities induced by chronic positive calorie excess have been only partially elucidated. We adopted a genome-wide approach to directly test whether long-term high-fat diet (HFD) exposure affects the DNA methylation profile of the mouse adipose tissue and to identify the functional consequences of these changes.

SUBJECTS/METHODS: We have used epididymal fat of mice fed either high-fat (HFD) or regular chow (STD) diet for 5 months and performed genome-wide DNA methylation analyses by methylated DNA immunoprecipitation sequencing (MeDIP-seq). Mouse Homeobox (*Hox*) Gene DNA Methylation PCR, RT-qPCR and bisulphite sequencing analyses were then performed.

RESULTS: Mice fed the HFD progressively expanded their adipose mass accompanied by a significant decrease in glucose tolerance ($P < 0.001$) and insulin sensitivity ($P < 0.05$). MeDIP-seq data analysis revealed a uniform distribution of differentially methylated regions (DMR) through the entire adipocyte genome, with a higher number of hypermethylated regions in HFD mice ($P < 0.005$). This different methylation profile was accompanied by increased expression of the *Dnmt3a* DNA methyltransferase (Dnmt; $P < 0.05$) and the methyl-CpG-binding domain protein *Mbd3* ($P < 0.05$) genes in HFD mice. Gene ontology analysis revealed that, in the HFD-treated mice, the *Hox* family of development genes was highly enriched in differentially methylated genes ($P = 0.008$). To validate this finding, *Hoxa5*, which is implicated in fat tissue differentiation and remodeling, has been selected and analyzed by bisulphite sequencing, confirming hypermethylation in the adipose tissue from the HFD mice. *Hoxa5* hypermethylation was associated with downregulation of *Hoxa5* mRNA and protein expression. Feeding animals previously exposed to the HFD with a standard chow diet for two further months improved the metabolic phenotype of the animals, accompanied by return of *Hoxa5* methylation and expression levels ($P < 0.05$) to values similar to those of the control mice maintained under standard chow.

CONCLUSIONS: HFD induces adipose tissue abnormalities accompanied by epigenetic changes at the *Hoxa5* adipose tissue remodeling gene.

International Journal of Obesity (2016) **40,** 929–937; doi:10.1038/ijo.2016.36

INTRODUCTION

Through the second half of the past century, obesity established as an increasingly prevalent response to the unhealthy environment and nutrition, both in the developed and in developing countries.[1] Obesity is associated with increased risk of a number of different metabolic complications, and with increased mortality.[2] In part, the excess morbidity accompanying obesity is determined by dysfunctional adipose tissue.[3]

While most caloric excess is stored in the subcutaneous adipose tissue, chronically positive caloric balance may induce ectopic storage of lipids and involve intra-abdominal, pericardial, perivascular and intramuscular adipose tissue (visceral adipose tissue). Increased visceral adipose tissue is well recognized as an independent risk factor for most obesity-associated comorbidities, with the distribution of adipose tissue, their anatomical, cellular and molecular features determining their occurrence.[4] However, it has been proposed that visceral adiposity represents a surrogate marker for global body fat dysfunction. [3]

Recent genome-wide association studies in humans led to the identification of >100 common gene variants determining individual propensity to adiposity. Most of them have an unknown function at the present.[5] In addition, the effect size of these variants is small compared with the large effect of unhealthy nutrition on the development of obesity.[6] Even in the case of this effect, however, the genomic bases of the visceral fat abnormalities induced by chronically positive calorie excess have been only partially elucidated.

It has become progressively clearer that environmental factors, including the quality of nutrition, may affect individual phenotypes by causing epigenetic modifications of the DNA.[7,8] These occur in the absence of DNA sequence alterations and include variations in the methylation status at specific regions of the genome, as well as posttranslational histone changes and

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Received 27 July 2015; revised 28 December 2015; accepted 24 January 2016; accepted article preview online 16 March 2016; advance online publication, 12 April 2016

differential miRNA expression.[9,10] Studies in humans have also revealed that specific epigenotypes have a very large effect on adiposity variance. Godfrey and co-workers[11] reported a positive correlation between the degree of *RXRa* methylation at one specific site in the promoter and childhood body adiposity at age 6 and 9 years. These findings were replicated in both within-cohort and between-cohort replicates. In contrast to the small effect sizes typically seen with genetic polymorphisms associated with metabolic disease risk, this single site epigenotype accounted for at least 25% of the variance in childhood adiposity. However, genes for which polymorphisms have strong associations with disease risk might be anticipated to have epimutations.[12] One such gene is *FTO*, that acts as a nucleic acid demethylase,[13] a co-substrate of which is α-ketoglutarate (a product of the Krebs cycle), recently described as a biomarker of obesity-associated non-alcoholic fatty liver disease.[14] The *FTO* gene has been first identified by genome-wide association studies as being involved in the risk of obesity. In a prospective study of nonsymptomatic individuals of a mean age of 30 years, Toperoff *et al.*[15] showed that those who featured hypomethylation at an intronic CpG site at *FTO* had an increased risk of developing impaired glucose metabolism.

However, the majority of these studies have focused on methylation at specific genes rather than adopting an unbiased approach, although functionally relevant DNA methylation changes may be located in different areas of the genome.[16,17] Thus, a comprehensive analysis of the genome methylation profile in response to changes in the nutritional status is necessary to interpret the epigenetic mechanisms accompanying diet-induced obesity.

In this work, we have adopted methylated DNA immunoprecipitation sequencing (MeDIP-seq) to identify whole-genome methylation changes occurring in the adipose tissue of mice exposed to a long-term treatment with fat-enriched diet. We have identified and cataloged several differentially methylated genes (DMGs) whose function is modulated by this nutritional regimen. We have further demonstrated that return of the animals to a standard chow diet caused improvement of the metabolic derangement, accompanied by rescue of both the epigenetic modifications and the function of selected genes. Some of them might therefore enable to quantify individual response to lifestyle intervention in humans as well as in mice.

MATERIALS AND METHODS

Animals and diet

Male 5-week-old C57BL/6 J mice were obtained from Charles Rivers (Deisenhofen, Germany) and housed in a temperature-controlled room (22 ± 2 °C) with 12- h light-dark cycle. Mice were enabled *ad libitum* access to food and water. Animals were randomly assigned to a standard chow diet (STD about 10% energy as fat) or a high-fat diet (HFD about 60% energy as fat) for 5 months. The composition of these diets is reported in Supplementary Table S1. Body weight was recorded weekly. For insulin tolerance testing, mice were fasted for 4 h and then subjected to intraperitoneal injection with insulin (0.75 mIU g^{-1} of body weight). Venous blood was subsequently drawn by tail clipping at 0, 15, 30, 45, 60, 90 and 120 min as previously described.[18] For intraperitoneal glucose tolerance testing, mice were fasted overnight and then subjected to intraperitoneal injection with glucose (2.0 g kg^{-1} of body weight). Venous blood was subsequently drawn by tail clipping at 0, 15, 30, 45, 60, 90 and 120 min as previously described.[18] Blood glucose levels were measured with Accu-Chek glucometers (Roche Applied Science, Penzberg, Germay). The total area under the curve and the inverse area under the curve for glucose response during glucose tolerance testing and insulin tolerance testing were calculated as previously described.[18] At the end of the experiment, the animals were killed; the epididymal white adipose tissue (WAT) was carefully dissected and stored at −80 °C until analyzed. For histology, epididymal WAT was fixed in 10% neutral buffered formalin and embedded in paraffin. Sections of 5 μm were obtained and stained with hematoxylin and eosin. Images captured by an Olympus BX51 microscope equipped with an Olympus (Shinjuku, Japan) DP-21 digital camera were processed using the ImageJ software.

All the procedures performed were in agreement with the National and Institutional Guidelines of the Animal Care and Use Committee at the University of Naples Medical School.

Fractionation of adipose tissue

WAT was washed and minced in Dulbecco's modified Eagle's medium and then incubated for 1 h at 37 °C with the same medium containing collagenase (1 mg ml^{-1}; Sigma–Aldrich, St Louis, MO, USA). The mixture was passed through a nylon filter (pore size, 250 μm) to remove undigested material. The filtrate was centrifuged for 5 min at 1500 *g* and floating cells and the pellet were recovered as the mature adipocyte fraction and the stromal vascular fraction, respectively, and were used for RNA extraction.

DNA preparation and MeDIP-Seq

Genomic DNA was isolated from WAT of STD and HFD mice using the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden-Düsseldorf, Germany) according to the manufacturer's instructions. The quality of each DNA sample was analyzed for integrity, purity and concentration on a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Purified DNA (>50 ng per sample) was then sent to Beijing Genomics Institute (BGI at Shenzhen, China) for MeDIP-seq analysis by a Illumina HiSeq 2000 (Illumina Inc., San Diego, CA, USA), and their detailed protocol was published in the study by Li *et al.*[19] DNA methylation data have been deposited in GEO database under accession number GSE71476.

Reads alignment and pre-processing

Data filtering was applied to remove adapter sequences, contamination and low-quality reads from raw reads. About 73 million of filtered fragments—36.5 million 50 bp paired-end reads—were obtained for each sample from MeDIP-seq experiments.

FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to assess the quality of sequenced reads. Raw reads were aligned to the reference mouse genome (UCSC mm9) using BWA[20] with default parameters for paired-end mapping. Reads alignments in SAM format were converted to BAM for further analyses using SAMtools.[20] Statistics of mapping quality was assessed by SAMStat on SAM/BAM input files. Nucleotide composition, length distribution, base quality distribution, mapping statistics, mismatches, insertions/deletions and error profiles were reported in html5 format including unmapped, poorly and accurately mapped reads. Only uniquely mapped reads were extracted from the. sam file by a custom shell script and were used for further analyses. BEDtools were used first to convert mapped reads in BED format and then to create files in Bedgraph format (.wiggle). These files were uploaded in UCSC Genome Browser (http://genome.ucsc.edu) for visual inspection and for gene-specific analysis.

Peak calling and identification of differentially methylated regions (DMRs)

The distribution of MeDIP-Seq reads on each mm9 chromosome was analyzed. The genome was divided into windows of the arbitrary 10 kb length, and read depth for each window was calculated. Read count within each window was normalized using the following equation: RC*10^6/UMR (RC = Read count in the 10 kb window; UMR = uniquely mapped reads). Results are shown in Supplementary Figure S1. Reads coverage analysis has been performed using BEDtools[21] to test the number of CpGs covered by sequenced reads, looking at the depth of coverage. The global distribution of MeDIP-Seq-generated reads on specific genomic elements of mouse genome (CGIs, shores and shelves, exons, introns, CDS, 5' and 3' untranslated regions (UTRs), as well as repeat elements) has been independently evaluated for both samples (Supplementary File S1). Annotations of the genomic elements of interest were downloaded from the Table Browser of UCSC Genome Browser for mm9 genome release. Genomic coordinates of CGIs in mm9 genome release were obtained from *Smallwood et al.*[22] Shores and shelves genomic coordinates were automatically computed from CGIs coordinates of.[22] We annotated CpG shores considering a range of ± 2 kb from the start and end positions of each CGI, and CpG shelves in the range of ± 2 kb from start and end of each CpG shore. MeDIP-Seq datasets from HFD and STD mice groups were independently analyzed with model-based analysis of ChIP-Seq[23] to find

significant methylation peaks in both samples, and with the MEDIPS package[21] to identify DMRs. For both samples, the *P*-value $< 10^{-10}$ was used to select significant peaks computed by model-based analysis of ChIP-Seq. Using MEDIPS package, uniquely mapped reads were extended in the sequencing direction to a length of 300 nt. For the identification of DMRs, we used the MEDIPS option that enables to compute differential methylation within fixed genome-wide frames. Mean r.p.m. (reads per million) values were computed for genome-wide 500 bp windows overlapping of 250 bp using MEDIPS ('frame_size = 500' and 'step = 250' parameters were used). DMRs calculated by the MEDIPS software were converted with custom scripts in BED format and loaded on Genome Browser for immediate visualization. DMRs were mapped to known genes or gene-associated elements (CGIs, shores and shelves, exons, introns, CDS, 5' and 3' UTRs, repeat elements) using SAMtools.[20] BED files of each annotated gene (or gene-associated elements) were downloaded from the Table Browser of UCSC. ChipPeakAnno was used to assign each CGIs to RefSeq genes based on the proximity of each gene transcription start site to the nearest CGI. A similar approach was extended to associate all DMRs to the above-mentioned gene-related elements. Protein Analysis THrough Evolutionary Relationships (PANTHER) Classification System (http://www.pantherdb.org) was used to determine whether hypermethylated CGIs are mostly associated with specific gene pathways and ontology terms. An false discovery rate < 0.05 was considered significant.

Bisulphite sequencing analysis

Genomic DNA bisulphite modification was performed using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instruction. The following primer pairs were designed by *Methprimer*[24] to amplify DMR-associated *Hoxa5* locus: sense 5'-TTGGA GTTGTTTAGGGAGTTTTTT-3'; antisense 5'-CCTCTAAAAATCATCAAACAA AATTTAC-3'. PCR products were purified using Min Elute Gel Extraction Kit (Qiagen) and cloned into the pGEM T-Easy vector (Promega, Fitchburg, WI, USA). Individual clones were grown and plasmids purified using the NucleoSpin Plasmid Kit (Macherey-Nagel, Düren, Germany). For each condition, 10 clones were sequenced using SP6 promoter primer on AB 3500 genetic analyzer (Applied Biosystem, Waltham, MA, USA). The proportion of methylation for each individual was calculated by dividing the total number of methylated sites in all clones by the total number of CG sites.

Methylated DNA immunoprecipitation (MeDIP)

MeDIP assay was performed as described by Weber *et al*.[25] Sonicated pooled genomic DNA from WAT was immunoprecipitated using anti-5meCpG (#ab10805, Abcam, Cambridge, MA, USA) or mouse IgG with anti-mouse IgG beads (#10003D, Life Technologies, Waltham, MA, USA). DNA methylation enrichment on recovered DNA was evaluated by qPCR. Samples were normalized to their respective input using the $2^{-\Delta CT}$ method. The following primer sequences were used: sense 5′-GTGCTTGATTTGTGGCTCGC-3′; antisense 5′-TCCACCCAACTCCCCCATTA-3′.

RNA isolation, reverse transcription and quantitative RT-PCR Analysis

Total RNA was isolated from WAT using AllPrep DNA/RNA Mini Kit (Qiagen). cDNA was synthesized by reverse transcription using Superscript III Reverse Transcriptase (Invitrogen, Waltham, MA, USA), according to the manufacturer's protocol. Real-time RT-qPCR was performed on an iCycler IQ multicolor Real Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using the comparative CT method (2-DDCT) with a Platinum SYBR Green qPCR Super-UDG mix (Bio-Rad Laboratories, Inc.; [26]). All reactions were performed in triplicate, and the relative mRNA expression levels of target genes were normalized to *cyclophilin* A. The primer sequences used are shown in Supplementary Table S2.

Mouse *Hox* Genes DNA methylation PCR array

The Mouse *Homeobox (Hox)* Genes DNA Methylation PCR Array (SABiosciences, Hilden-Düsseldorf, Germany) has been used to profile DNA methylation levels of *Hox* genes ($n = 22$). This novel restriction enzyme-based technology was described by *Kim et al*.[27] Briefly, genomic DNA is treated with a combination of methylation-sensitive and/or methylation-dependent enzymes. Subsequent RT-qPCR enabled comparison of Ct values between particular enzymatic reactions and thus assessment of DNA methylation levels. PCR reactions were performed using iCycler IQ multicolor Real Time PCR Detection System (Bio-Rad Laboratories, Inc.). Detailed description of sample preparation and PCR reaction conditions are provided in the manufacturer's protocol.

Western blot analyses

Adipose tissues were lysed in T-PER reagent (#78510, Thermo-Scientific, Waltham, MA, USA) with a protease inhibitor cocktail (#05892791001, Roche) and a phosphatase inhibitor (#04906837001, Roche). Twenty micrograms of protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Upon incubation with primary Hoxa5 (1:1000; #ab82645, Abcam), α-tubulin (1:1000; #SC5546, Santa Cruz, Dallas, TX, USA) and secondary antibodies (Bio-Rad Laboratories, Inc.), immunoreactive bands were detected by enhanced chemiluminescence according to the manufacturer's instructions (Pearce ECL Western Blotting Substrate, # 32106, Thermo-Scientific).

Cell culture, treatments and transfection

3T3-L1 cells were grown and were allowed to differentiate into mature adipocytes as described Bezy *et al*.[28] Subsequently, adipocytes were exposed to palmitate (0.25 mM; Sigma–Aldrich) and harvested 96 h later for DNA and RNA extraction. For *Hoxa5* silencing during adipogenesis, 3T3-L1 cells were transfected with 25 nmol l^{-1} of *Hoxa5* siRNA (OriGene, Rockville, MD, USA), and subsequently induced to differentiate into mature adipocytes. Transfection was repeated as on day − 2 every 48 h until day 8. Then, lipid accumulation was assessed by Oil Red O staining as previously described.[29]

Statistical analysis

Data are expressed as mean ± s.e.m. Comparison between groups were performed using Student's *t*-test or the one-way analysis of variance followed by Tukey multiple comparison tests, as appropriate, using GraphPad Software (version 6.00 for Windows, San Diego, CA, USA): a *P*-value $\leqslant 0.05$ was considered significant. Statistical significance of DMRs—in HFD compared with STD mice—in genomic regions was performed with chi-square test.

RESULTS

HFD changes the global DNA methylation profile in adipose tissue from C57BL/6 J mice

Five-week-old male mice were fed either standard chow (STD) or fat-enriched diets (HFD) for 5 months followed by metabolic phenotyping. Despite comparable food intake in the two groups, mice exposed to the HFD exhibited a 37% increase in body weight ($P < 0.001$). Fasting glucose levels were increased in the HFD treated mice ($P < 0.05$). Glucose and insulin tolerance tests revealed, respectively, decreased glucose tolerance ($P < 0.001$) and insulin sensitivity ($P < 0.05$) in these animals (Table 1).

HFD treatment induced an increase in the individual adipose cell size, accompanied by an elevated expression of macrophage marker genes, including *F4/80*, *Cd68* and *Mcp-1*. Moreover, HFD exposure determined an upregulation of *Fabp4* and *Fas* mRNA

Table 1. Metabolic phenotypes of high-fat- and standard chow diet-exposed mice

	STD (n = *16*)	*HFD* (n = *16*)	P-*value*
Body weight	30.78 ± 0.9	42.18 ± 1.1	$P < 0.001$
AUC GTT (mg dl^{-1}*120 min)	11150 ± 1463	23686 ± 1783	$P < 0.001$
AUC inv. ITT (mg dl^{-1}*120 min)	10986 ± 920.12	6702.85 ± 1136.34	$P < 0.05$
Glucose (mg dl^{-1})	106 ± 12.7	163 ± 14.1	$P < 0.05$

Abbreviations: AUC, area under the curve; GTT, glucose test tolerance; ITT, insulin test tolerance; HFD, high-fat diet mice; STD, standard diet mice. All values are means ± s.e.m.

levels, together with a significant reduction of adiponectin mRNA levels, whereas *Ppary2* expression showed no significant variation between HFD- and STD-fed mice, similar as described previously[29,30] (Supplementary Figure S1).

We have subsequently compared global methylation profiles in epididymal fat from STD- and HFD-treated animals. By immunoprecipitation of methylated DNA followed by massively parallel sequencing (MeDIP-seq[25,31]), we generated 73 million reads in each condition. Alignment to the mouse genome (mm9) revealed that about 50% of the reads mapped in unique regions (Supplementary Table S3). In addition, these reads were uniformly distributed through the entire mouse genome, as indicated by the detection of methylation events in most chromosomal regions (Supplementary Figure S2), and were found to be mainly located within introns and exons regions of the gene-associated elements (Supplementary Table S4A).

About 17.6×10^4 and 16.7×10^4 methylation peaks were identified, respectively, in HFD- and STD-maintained mice (Bonferroni corrected $P<0.01$). Most of these peaks (52%) were within the gene bodies, with 36% in intronic regions and 9% in CpG islands (Supplementary Table S4B). Analysis of different repeat types revealed that methylation peaks were abundant in *LTR* and *LINE* classes of the mouse genome (Supplementary Table S4C).

About 14.8×10^3 DMRs were also identified in the genomes from HFD- and STD-exposed mice ($P<0.005$), about 53% of which were hypermethylated while 47% were hypomethylated in the HFD compared with the STD mice. The highest frequency of DMR occurrence was detected in gene bodies, particularly in the intronic sequences where hypermethylation events were more common (Figure 1a). Significant DMR hypermethylation was also noted in the CpG islands of HFD-fed mice, while no significant difference was observed in the CpG shores and shelves (Figure 1b). At variance with the gene-associated elements, repeat type analysis identified an increased number of hypomethylated DMR in *LTR* and *LINE* classes of the HFD mouse genome (Supplementary Figure S3).

Effect of HFD on expression of DNA-methylating enzymes in adipose tissue

We then sought to explore whether the changes in DNA methylation profile observed in mice exposed to HFD could be attributed to changes in the expression of DNA-methylating enzymes and focused our attention on DNA methyltransferases[32,33] and methyl-CpG-binding domain proteins (Mbd[34]). Interestingly, quantitative real-time PCR analysis of mRNA from adipose tissue revealed a specific twofold increase in the abundance of *Dnmt3a* transcript in the HFD-fed mice ($P<0.05$; Figure 2a). The *Mbd3,* but not the *Mbd1, Mbd2* or *Mbd4* genes was also significantly overexpressed in the adipose tissue of HFD-fed mice ($P<0.05$; Figure 2b), suggesting a role *Dnmt3a* and *Mbd3* genes in mediating diet-induced hypermethylation events.

Diet-induced methylation changes affect developmental processes

For the purpose of the present study, genes whose nearest CpG island, shelf and/or shore overlap a DMR were termed differentially methylated genes, which allowed us to identify 1.673×10^3 DMG. To initially address their functional significance, we performed gene set enrichment analysis both for the gene ontology categories and the canonical pathways.

This analysis enabled us to identify the '*Ppar signaling pathway*' as the most significantly enriched pathway among DMG (false discovery rate < 5%; Supplementary Table S5). DMG were also enriched in the '*PI3k-Akt*', '*Insulin resistance*', '*Cytokine-cytokine*

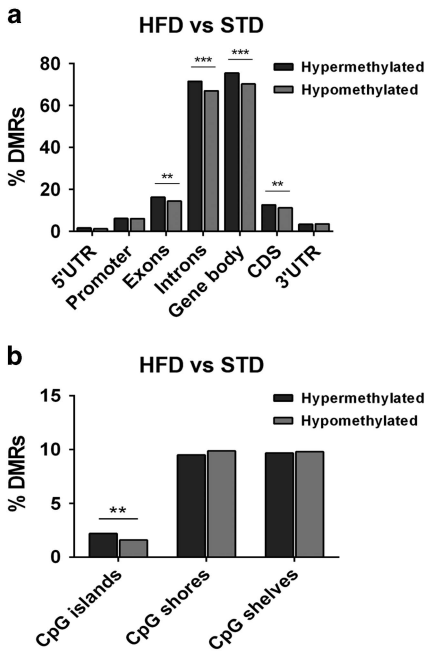

Figure 1. DMR distribution in mice subjected to HFD feeding. Distribution of hypermethylated and hypomethylated DMRs (**a**) across different gene elements (promoter, 5-UTR, exons, cds, introns, 3-UTR and gene body) and (**b**) CpG-Island, -Shores and -Shelves. A CpG-Island is defined 200 bp (or larger) fragment of DNA with a C+G content of >50% and an observed CpG/expected CpG ratio > of 0.6. Shores: the flanking regions of the CpG-Islands, 0–2000 bp. Shelves: the regions flanking the island shores, i.e., covering 2000–4000 bp distant from the CpG-Island. $^{**}P<0.005$ and $^{***}P<0.0005$.

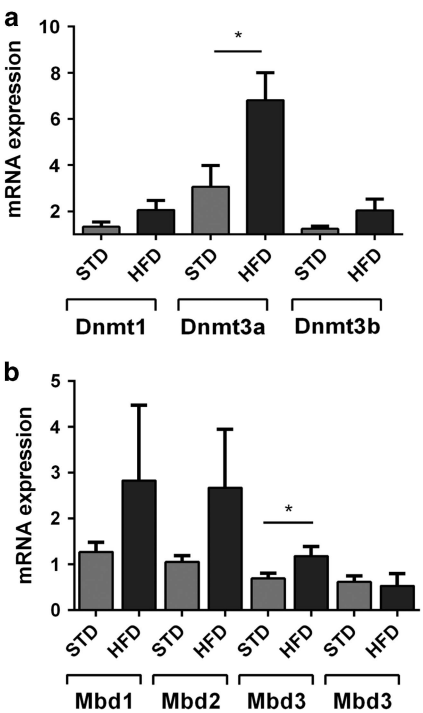

Figure 2. Expression of *Dnmt* and *Mbd* in WAT from HFD-exposed mice. qRT-PCR analysis of mRNA expression levels of WAT *Dnmt1, Dnmt3a, Dnmt3b* (**a**), and *Mbd1*, *Mbd2*, *Mbd3* and *Mbd4* (**b**). All data are expressed as means ± s.e.m. ($n=8$ for STD; $n=8$ for HFD). $^{*}P<0.05$, HFD *vs* STD.

receptor interaction' and '*MAPK*' pathways (false discovery rate < 5%; Supplementary Table S5A–D).

Gene ontology analysis indicated a strong enrichment in genes involved in metabolic, transcription and developmental processes in samples from HFD-exposed mice (Supplementary Figure S4). Interestingly, a distinct set of genes belonging to the *Hox* family of transcription factors was highly significantly enriched among DMG ($P = 0.008$). Aberrant methylation of transcription factor promoters induce a wide array of downstream effects on gene expression. In addition, increasing evidence indicate a role of *Hox* genes in adipose tissue function and metabolic disorders.[35] Therefore, we focused on this cluster of genes for further investigation. We have adopted a DNA methylation PCR array and systematically examined the promoter methylation of 22 *Hox* genes. Whereas most of these genes featured no significant methylation differences in DNA from HFD and STD-exposed mice, *Hoxa5* and *Hoxa11* featured significant promoter hypermethylation in DNA from HFD compared with STD-fed mice (Figure 3a). At variance, *Hoxb1, Hoxb9, Hoxc6* and *Hoxc8* showed reduced methylation in the HFD-exposed animals.

Increased DNA methylation at the 5'-UTR promoter region is often accompanied by transcriptional repression, whereas activation commonly follows hypomethylation.[36] To directly test the significance of the observed methylation changes in *Hox* genes,

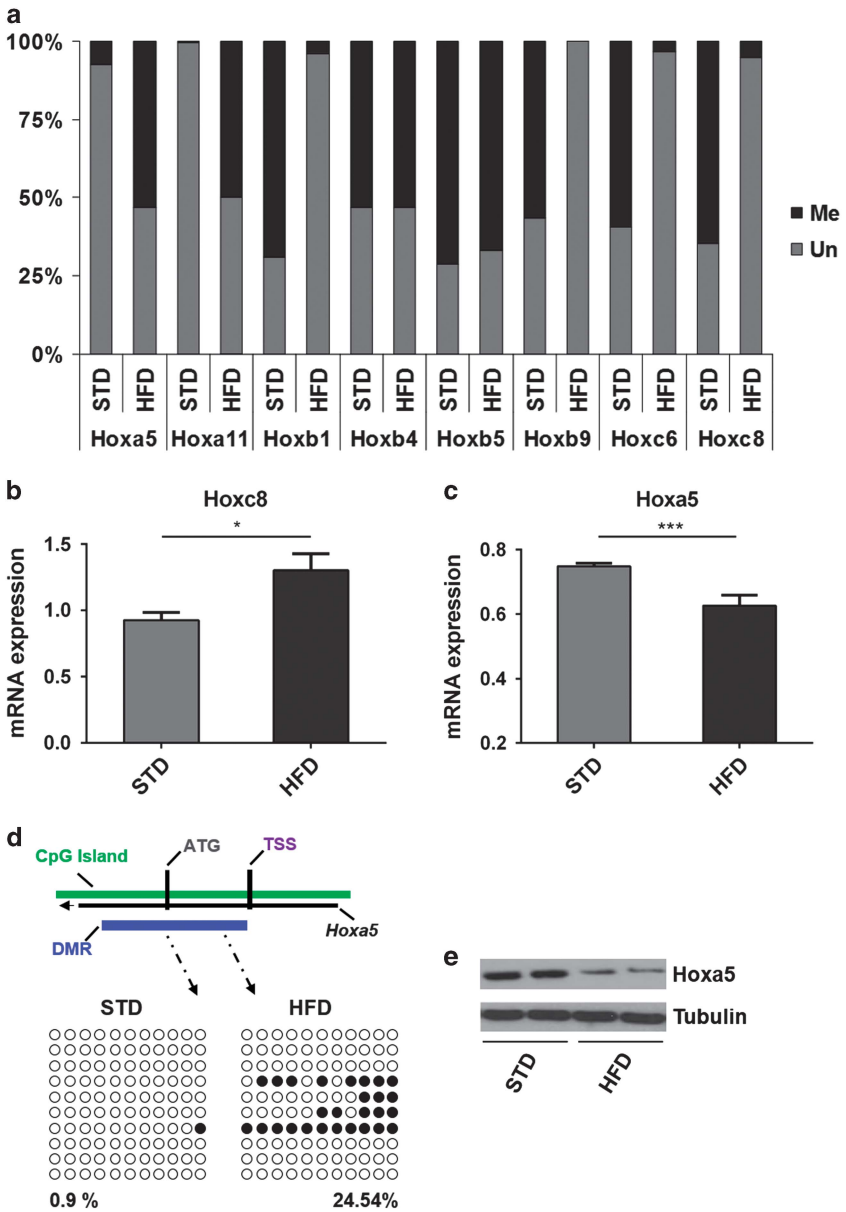

Figure 3. (**a**) DNA methylation and expression levels of *Hox* genes in WAT from HFD and STD-treated mice. A mouse Hox gene DNA methylation array was used to profile the indicated Hox methylation levels as described under Materials and methods. Un, unmethylated; Me, methylated. RT-qPCR analysis of mRNA expression levels of WAT *Hoxc8* (**b**) and *Hoxa5* (**c**). All mRNA expression data are expressed as means ± s.e.m. ($n = 8$ for STD; $n = 8$ for HFD). (**d**) MeDIP-seq signals at *Hoxa5* gene locus and bisulfite analysis of the *Hoxa5*-associated DMR. Blue bar indicates hypermethylated DMR at *Hoxa5* locus in HFD. CpG-Island (in green) overlapping *Hoxa5* locus (in black). Start codon ATG (gray) and transcription start (purple) sites are also indicated. Graphic representation and results of the bisulphite-sequenced portion of the DMR (red arrows) relative to the *Hoxa5* locus. Each horizontal row represents a single clone; the methylation percentages of 10 individual clones are indicated, with unmethylated (○) and methylated (●) CpG sites. Representative image of $n = 3$ mice per group. (**e**) Immunoblot analysis of Hoxa5 protein level ($n = 2$); α-tubulin was used as a loading control. ***$P < 0.001$ and *$P < 0.05$.

we performed RT-qPCR assays. On the basis of this approach, *Hoxc8* mRNA levels in adipose tissue from HFD-exposed mice were found to be significantly higher compared with control mice (Figure 3b). At variance, *Hoxa5* mRNA levels were decreased in the adipose tissue of HFD-fed mice (Figure 3c). To further validate the HFD-induced methylation changes occurring at the *Hoxa5* gene, we have subjected to bisulphite treatment and then directly sequenced the *Hoxa5*-associated DMR, which encompasses a significant portion of the 5'-UTR promoter region of the *Hoxa5* gene. As shown in Figure 3d, this procedure revealed an average 25% increased methylation of the region from HFD compared with STD-exposed mice ($P < 0.01$), validating the MeDIP-seq data. Furthermore, the amounts of the Hoxa5 protein in WAT were decreased in the HFD-fed mice compared with controls (Figure 3e).

To investigate whether diet-induced obesity or specific fats is responsible for the changes in *Hoxa5* methylation and expression associated to exposure to HFD, we analyzed the effect of different fat components on *Hoxa5* in cultured 3T3-L1 cells. Supplementation of the culture medium with 0.25 mM palmitate, a major component of the HFD, increased *Hoxa5* promoter methylation and decreased the expression of the gene to a similar extent as that observed in WAT from HFD-treated mice (Supplementary Figure S5A and B). Medium supplementation with 0.25 mM oleate did not elicit any significant effect.

Reversibility of HFD effect on *Hoxa5* function

Current evidence indicates that, once established, epigenetic modifications may be persistent.[37] We therefore sought to assess the stability of HFD-induced changes in promoter methylation and expression of *Hoxa5*. To this end, we have prolonged the nutritional intervention in animals exposed to HFD by returning them to a STD for a further 2-month period, followed by re-assessment of the metabolic phenotype. This diet change was accompanied by a 50% reduction in the excess weight caused by the previous HFD regimen ($P < 0.01$; Figure 4a). Fasting plasma glucose levels and IP glucose tolerance test were also significantly improved, indicative of improved glucose tolerance (Figures 4b and c). Importantly, *Hoxa5*-associated DMR featured an almost twofold decreased global methylation (Figure 5a). This effect was accompanied by an almost complete rescue of *Hoxa5* mRNA expression in the adipose tissue (Figure 5b).

Expression of *Hoxa5* is modulated during *in vitro* adipogenesis

In HFD-fed mice, *Hoxa5* expression was downregulated in isolated adipocytes but remained unchanged in stromal vascular fraction cells (Supplementary Figure S6A). To investigate the role of *Hoxa5* in WAT biology, we first evaluated *Hoxa5* expression during adipogenesis, and found that *Hoxa5* mRNA was induced in parallel with differentiation in 3T3-L1 preadipocytes (Supplementary Figure S6B). Consistently, the expression of *Hoxa5* mRNA was higher in the adipocyte fraction than in WAT stromal vascular fraction from mice maintained under standard chow diet (Supplementary Figure S6A).

3T3-L1 cells were subsequently transfected with *Hoxa5*-specific siRNA and then induced to differentiate into mature adipocytes (Supplementary Figure S6C). Oil Red O staining revealed a decrease in lipid accumulation in the *Hoxa5*-siRNA treated compared with control cells (Figure 5c). In parallel, the expression of the adipogenic markers *Ppary2* and *C/ebpa* was reduced in *Hoxa5*-siRNA-treated cells (Figure 5d), suggesting that, at least in the 3T3-L1 cells, *Hoxa5* is important for adipocyte differentiation.

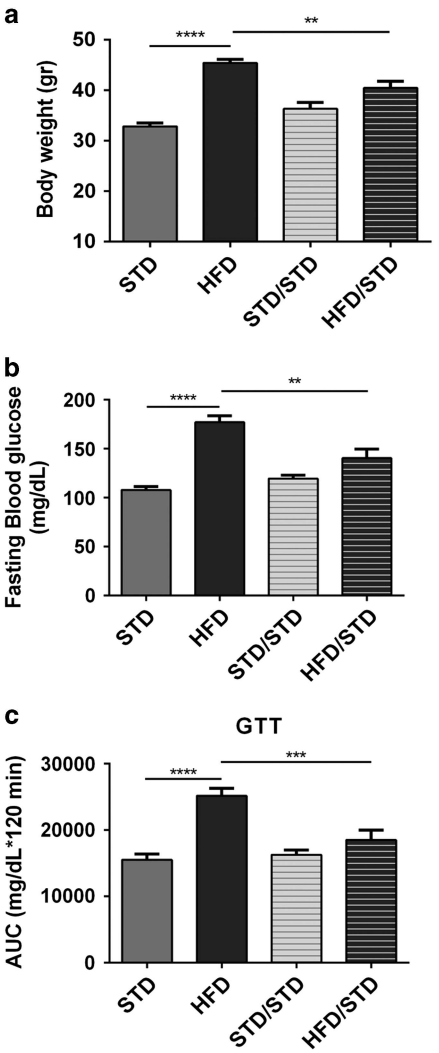

Figure 4. Metabolic parameters of HFD-treated mice upon STD rescue. Mice were either maintained under STD for 7 months (STD/STD) or returned (and maintained for two further months) to a STD after 5-month HFD treatment. Animals maintained under STD or HFD for 5 months (respectively, STD, HFD) are also shown for comparison. (**a**) Body weight; (**b**) fasting blood glucose; (**c**) glucose AUC during GTT. Data are means ± s.e.m. ($n = 8$ for STD; $n = 8$ for HFD; $n = 8$ for STD/STD; $n = 8$ for HFD/STD). $^{**}P < 0.01$ $^{***}P < 0.001$, $^{****}P < 0.0001$. STD/STD mice maintained on STD diet through the entire protocol; HFD/STD, HFD-treated mice returned to STD; AUC, area under the curve; GTT, glucose tolerance test.

DISCUSSION

We have adopted MeDIP-seq[25,31] analysis to directly test whether long-term HFD exposure affects the DNA methylation profile of the mouse adipose tissue and to identify the consequences of these changes. We have shown a greater number of hypermethylated compared with hypomethylated regions in the gene elements of the HFD-exposed animals indicating that, in association with obesity, this nutritional regimen induces increased DNA methylation in this part of the genome. Consistent with our finding, human obesity has been shown to be associated with gene element hypermethylation in the skeletal muscle.[38] In addition, very recent analysis of global DNA methylation profiles of adipose tissue from severely obese individuals showed increased methylation in the gene bodies and 3' UTRs compared with the same subjects examined upon bariatric surgery and weight loss.[39]

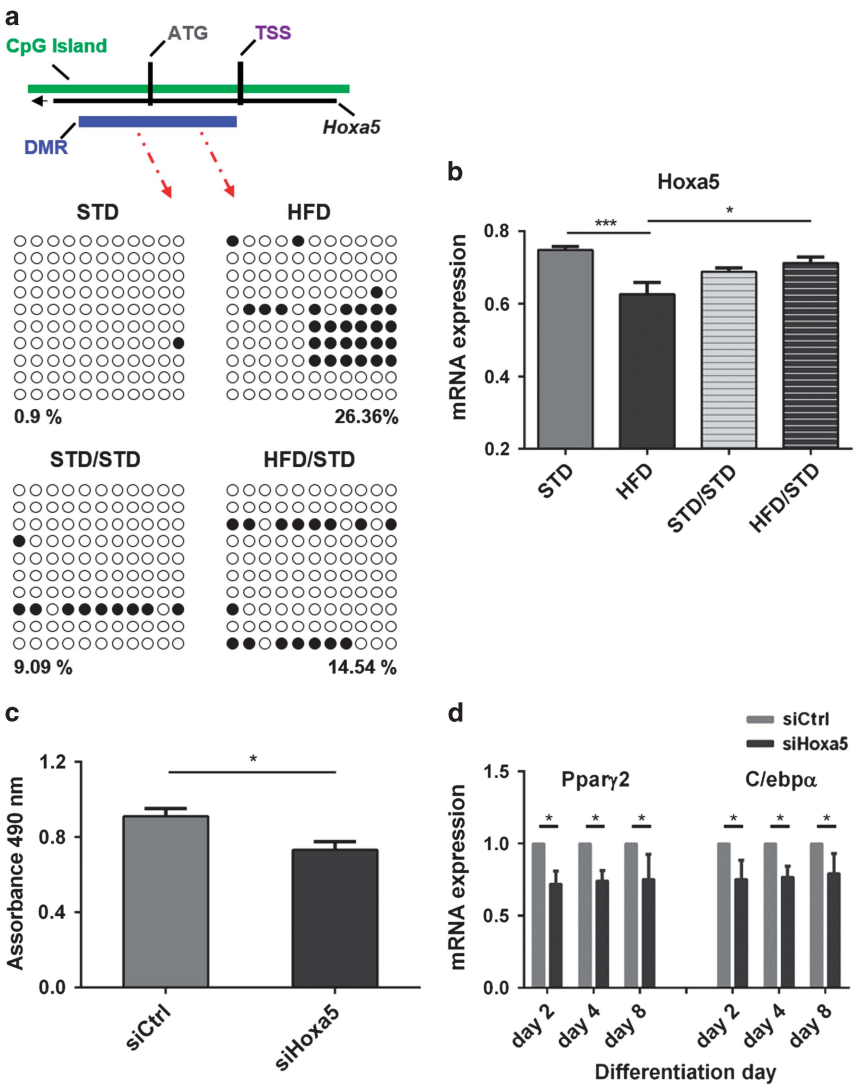

Figure 5. *Hoxa5* DMR methylation status and mRNA levels after STD rescue and the effect of *Hoxa5* silencing on adipogenesis *in vitro*. (**a**) Graphic representation of bisulphite sequencing of the *Hoxa5*-DMR in WAT from animals maintained under HFD or returned to a standard chow diet (STD). Each horizontal row represents a single clone; the methylation percentages of 10 individual clones are indicated, with unmethylated (○) and methylated (●) CpG sites. The data shown are representative of three mice per group. (**b**) qRT-PCR analysis of mRNA expression levels of WAT *Hoxa5* after returning the HFD mice to STD. Data are means ± s.e.m. ($n = 8$ for STD; $n = 8$ for HFD; $n = 8$ for STD/STD; $n = 8$ for HFD/STD). *$P < 0.05$ ***$P < 0.001$. (**c**) Effect of *Hoxa5* knockdown on the extent of adipocyte differentiation was assessed by Oil Red O staining of intracellular triglyceride, and measuring the extract's absorbance at 490 nm. (**d**) qRT-PCR analysis of mRNA expression levels of *Ppary2* and *C/ebpa* in 3T3-L1 treated with *Hoxa5*-specific siRNA and controls, at different time point during differentiation. All data are represented as means ± s.e.m. of triplicates. *$P < 0.05$.

Maunakea and co-workers[40,41] have recently reported that, in mammals, methylation occurring in the gene-associated regions mainly involve gene bodies rather than promoter 5' CGI, as < 3% of promoter-associated CGI are methylated. Similarly, our MeDIP-seq analysis revealed that most methylated sites identified in the mouse adipose tissue falls within the gene bodies, while methylation peaks at the 5'-UTR and gene promoter regions only account for a small fraction of the total number of the genome-wide methylated regions. Our data further revealed that only a small proportion of methylated CGI undergo methylation changes in response to HFD exposure, suggesting that CGI methylation is specifically affected by the nutritional intervention. In addition, despite the important role in gene regulation of the CGI shores and shelves,[42] our study did not reveal any significant difference in the methylation of these regions in response to HFD, indicating that CGI, rather than their surrounding regions, are involved in mediating the impact of the diet on genome plasticity.

At variance from the gene-associated elements, repeated elements, particularly the *LTR* and *LINE* families, are hypomethylated in the HFD mice. Interestingly, in mice, *LTR* hypomethylation is responsible for the agouti phenotype,[43] while in humans, *LINE* hypomethylation in the visceral adipose tissue is associated with increased risk of metabolic syndrome in the presence of obesity.[44] All together, these findings underline the potential importance of these repetitive elements in determining pathological traits in humans as well as in mouse models.

The level and pattern of DNA methylation are regulated by both DNA methyltransferases (DNMT1, DNMT3A and DNMT3B) and demethylating proteins.[45] In addition, the effects of DNA methylation on chromatin and gene expression are largely mediated by methylated DNA reader proteins, including Methyl-CpG-binding domain protein 3 (*Mbd3* (ref. 46)). In this study, we report that the increased methylation of several genomic regions in adipose tissue of HFD-exposed mice is accompanied by the

upregulation of *MBd3* and *Dnmt3a*. It is possible that a causal relationship exists between this increased *MBd3* and *Dnmt3a* expression and the observed hypermethylation at specific genes/gene-associated elements occurring in mice in response to HFD treatment. It is equally possible that the enhanced *MBd3* and *Dnmt3a* function impair the DNA demethylation machinery which is, in turn, responsible for the hypermethylation revealed in the HFD mice. Both mechanisms may also simultaneously occur. Interestingly, however, recent studies by Kohno and co-workers[47] have provided evidence for an important role of *Dnmt3a* in linking environmental determinants to altered energy homeostasis. Independent investigations have further identified evidence that this methylase is involved both in the development of obesity and in the obesity-related inflammation caused by HFD exposure.[48] Demethylation events also seemed to affect several genomic regions in the HFD-treated mice. The impact of these changes is presently under investigation in the laboratory, as available evidence indicates that they may well have effects on cell phenotype.[49]

Investigating the functional consequences of the genome-wide changes in DNA methylation accompanying HFD exposure led us to recognize that most DMG that we have identified encode for transcription factors. Interestingly, the class I Homeobox family of transcription factors (*Hox* genes[35]) revealed a very significant DMG enrichment in mice treated with HFD. In these same mice, a perturbation in the methylation of genes included into the *Ppar signaling, PI3K-AKT* and *insulin resistance* pathways was also detected. However, different considerations led us to focus our attention on the *Hox* genes in the context of the present work. First, besides the well-described role in controlling tissue regional development,[35] increasing evidence indicates that *Hox* genes are highly expressed in the adipose tissue[35] and have an active role in controlling adipocyte functions, including differentiation, and body fat distribution.[50,51] Second, human studies further revealed that different members of the *HOX* subfamily are upregulated upon the severe weight loss following bariatric surgery, indicating that they respond to changes in body fat mass.[39,52] Third, current evidence indicates epigenetic control of *Hox* genes during neurogenesis, development and disease.[53] Thus, the contribution of diet-induced epigenetic modifications to the control of *Hox* gene function in the adipose tissue deserved to be clarified. Indeed, an aberrant methylation of transcription factor promoters may potentially trigger a completely distinct transcriptional program in the hit tissues, producing a broad *spectrum* of downstream transcriptional events.

In this work, we have shown that long-term HFD exposure simultaneously changes methylation and expression of several *Hox* genes. Among these genes, *Hoxa5* exhibited the more robust repression in response to diet-induced hypermethylation. The transcriptional repression accompanying the epigenetic dysregulation of *Hoxa5* may have been, at least in part, caused by the saturated fat enrichment of the diet rather than representing a consequence of obesity, as it can be mimicked by exposing 3T3-L1 adipocytes to palmitate. It remains possible, however, that obesity *per se* or specific obesity-associated traits further contribute to *Hoxa5* dysregulation.

The downregulation of *Hoxa5* expression may contribute to the adipose tissue functional changes and remodeling that accompany weight gain determined by HFD exposure. Indeed, *HOXA5* expression increases during differentiation of human primary adipocytes and is differentially regulated in various adipose tissue districts.[54,55] Consistently, we report that the expression of *Hoxa5* is modulated during *in vitro* adipogenesis in 3T3-L1 and its mRNA levels were higher in the adipocyte fraction than in the stromal vascular fraction of WAT from lean mice. In addition, *Hoxa5* silencing was accompanied by an impaired differentiation, paralleled by an altered adipogenic gene expression and decreased lipid accumulation. These findings, and the crucial role of the *Hox* genes in developmental processes, highlight the potential role of *Hoxa5* as regulator of differentiation and/or commitment of adipogenic precursor cells.

Previous studies reported that *Hoxa5* inhibits angiogenesis,[56] while angiogenesis inhibitors improve obesity in animal models.[51] Blood vessels not only supply nutrients and oxygen to individual adipose cells, they also serve as a cellular reservoir to provide adipose precursor and stem cells that control adipose tissue mass and function.[57] It is possible therefore that HFD-induced downregulation of *Hoxa5* negatively affects recruitment of new adipose cell precursors or contribute to the adipose cell dysfunction that often accompany adipose tissue expansion in response to caloric excess.

HFD-exposed mice returned to a standard chow diet for a further 2-month period underwent simultaneous weight loss and almost complete rescue of *Hoxa5* mRNA levels. Importantly, *Hoxa5* methylation also returned to levels comparable with control animals fed the standard chow diet for an identical period of time. This finding supported the role of diet-induced *Hoxa5* methylation in controlling mRNA expression of this gene. Similar to the HFD-exposed mice, obese human individuals achieving profound weight loss after bariatric surgery treatment also revealed improved *HOXA5* expression in adipose tissue.[52] Whether quantification of *Hoxa5* expression can be exploited to assess response to caloric restriction in animals and in humans, is presently being investigated in the laboratory.

In conclusion, we have used MeDIP-seq analysis to verify whether the exposure to HFD modifies the DNA methylation profile in mouse adipose tissue and obtain indications on the consequence of these changes on adipocyte function. Our work reveals that the level of *Hoxa5* mRNA expression, whose function is implicated in fat tissue remodeling in response to changes in body fat mass, is repressed in association with the methylation changes caused by the nutritional intervention. Thus, environmental cues may generate adipose tissue dysfunction by inducing epigenetic modifications. Rescue of *Hoxa5* methylation and function in response to standard chow diet indicates that its expression may represent a potential tool to quantify obesity response to nutritional intervention.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

LP is the recipient of the EFSD/Lilly Research Fellowship 2015. This work has been supported, in part, by the European Foundation for the Study of Diabetes (EFSD), by the Ministero dell'Università e della Ricerca Scientifica (grants PRIN and FIRB-MERIT, and PON 01_02460) and by the Società Italiana di Diabetologia (SID-FO.DI.RI). This work was also supported by the P.O.R. Campania FSE 2007-2013, Project CREMe.

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Supplementary Information accompanies this paper on International Journal of Obesity website (http://www.nature.com/ijo)

J Endocrinol Invest (2016) 39:1095–1103
DOI 10.1007/s40618-016-0473-1

REVIEW

Epigenetics: spotlight on type 2 diabetes and obesity

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Received: 10 March 2016 / Accepted: 18 April 2016 / Published online: 14 May 2016

Abstract Type 2 diabetes (T2D) and obesity are the major public health problems. Substantial efforts have been made to define loci and variants contributing to the individual risk of these disorders. However, the overall risk explained by genetic variation is very modest. Epigenetics is one of the fastest growing research areas in biomedicine as changes in the epigenome are involved in many biological processes, impact on the risk for several complex diseases including diabetes and may explain susceptibility. In this review, we focus on the role of DNA methylation in contributing to the risk of T2D and obesity.

Keywords Epigenetics · DNA methylation · Type 2 diabetes · Obesity

Epigenetics: current status of knowledge

It is now well recognized that environmental factors including diet, physical activity, drugs and smoking, affect the phenotype and provide a major contribution to susceptibility to most chronic non communicable diseases [1]. Epigenetics acts at the interface between the genome and environmental factors, and might be broadly defined as the sum of all the mechanisms necessary to unfold the genetic program into development [2]. In the early 1940 s, Conrad Waddington linked genetics and developmental biology coining the term epigenetics. He defined epigenetics as "the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being" [3]. However, the meaning of the word has gradually changed over the following years, and epigenetics is known today as "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence" [4]. Differently from traditional genetics, based on cell lineages and clonal inheritance, epigenetic changes often occur in groups of cells while some epigenetic events are clonal. In addition, genetic changes are, almost by definition, stable, whereas epigenetic changes are plastic events [2]. An example of the latter concept is provided by genomic imprinting, where DNA methylation may be lost during development, or when persisting, it is erased and re-setted during gametogenesis [5]. Epigenetic mechanisms are plastic genomic processes that change genome function under endogenous and exogenous influences [6, 7], and may propagate modifications of gene activity from one cell generation to the next [8]. These mechanisms imply chemical modification of DNA, such as DNA methylation, post-traslational changes in histone proteins altering chromatin conformation, and transcriptional gene silencing mediated by non-coding RNAs (ncRNAs) [9] (Fig. 1). Abnormalities in one or more of these mechanisms can lead to inappropriate expression or silencing of genes, resulting in imbalance of the epigenetic network and may result in metabolic disorders such as T2D and obesity [10, 11].

Epigenetic mechanisms and gene function

DNA methylation

DNA methylation is a covalent modification of DNA that occurs at position 5 of the cytosine pyrimidine ring [12].

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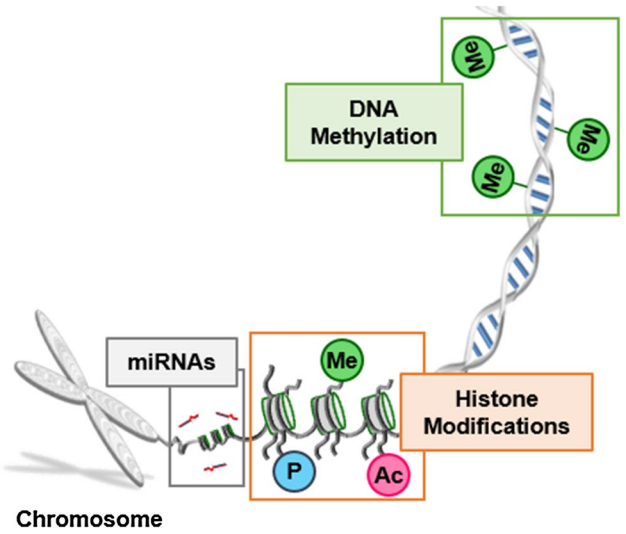

Fig. 1 Schematic representation of epigenetic modifications. Epigenetic modifications include DNA methylation, histone modifications and miRNAs. DNA methylation preferentially occurs at CpG di-nucleotides and is generally recognized as an epigenetic silencing mechanism. Histone modifications, instead, influence gene expression by directly altering the chromatin conformation through the passage from a condensed transcriptionally silent heterochromatin to transcriptionally active euchromatin and vice versa. Different types of modifications are known and include acetylation (Ac), methylation (Me), and phosphorylation (P) of histones tails. Finally, miRNAs act at post-transcriptional level by suppressing target gene expression or, through a non-perfect complementarity between miRNA and target mRNA that causes translation inhibition of the target or through near-perfect complementarity which results in the degradation of the target mRNA

Nearly four decades ago, DNA methylation was identified as hotspot for spontaneous base substitutions [13]. Indeed, while spontaneous deamination of cytosine produces uracil, a nitrogenous base that does not belong to DNA and that is immediately recognized and corrected by the system of DNA repair; deamination of 5-methyl cytosine produces thymine, causes C:G to T:A transitions, and creates a mismatch that the system of DNA repair does not always preserve [10, 14]. DNA methylation is the better characterized epigenetic mark. In mammals, it is essential during development and is involved in a variety of biological processes, including genomic imprinting and X chromosome inactivation [15]. DNA methylation is established during embryogenesis by de-methylation and de novo methylation events that can be inherited and maintained clonally by the action of specific enzymes termed DNA methyltransferases (DNMTs) [15, 16]. DNMT1 faithfully and symmetrically propagates cytosine methylation through recognition of methylated cytosines from an existing DNA strand to its novel partner upon replication and is primarily responsible for the maintenance of DNA methylation in cells. DNMT3A and DNMT3B are mainly involved in de novo methylation and establish new methylation patterns [4]. DNA methylation has long been recognized as an epigenetic silencing mechanism [17] which preferentially occurs at CpG di-nucleotides that are usually clustered in the CpG islands (CGIs) [18]. Quite often, un-methylated CpG sites at gene promoters create a transcriptionally permissive chromatin state by destabilizing nucleosomes and facilitating the recruitment of transcription factors [19]. On the other hand, dense DNA methylation of CpGs mediates stable long-term gene silencing by direct inhibition of transcription factors binding or by a combination of events mediated by methyl-CpG binding domain proteins (MBDs) which recruit methylated DNA mediators of chromatin remodeling, such as histone deacetylases (HDACs), or other repressors of gene expression (Table 1) [17, 20, 21].

Histone modifications

In eukaryotic cells, nucleosomes are the repeating and functional structural units of chromatin. They are composed of DNA wrapped around eight histone proteins, two homo-dimers histones H3 and H4, and two hetero-dimers histones H2A/H2B. Nucleosomes are mutually connected among themselves by the stretches of variable length of DNA linker conveyed by histone H1 [22, 23]. Histone modifications, such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation and ADP ribosylation, are reversible epigenetic modifications, occurring at the histone tails. These modifications regulate gene expression by dynamically altering chromatin conformation causing electrostatic change and/or modulating binding proteins to chromatin [22, 24]. Even more extensively than other types of modifications, acetylation and methylation mediate formation of the condensed transcriptionally silent heterochromatin and of the transcriptionally active euchromatin. In mammalian cells, heterochromatin prevails and is generally characterized by high levels of DNA methylation and histone de-acetylation, and is enriched in tri-methylation of H3-Lys9, H3-Lys27, and H4-Lys20 [25, 26]. On the other hand, euchromatin exhibits lower levels of DNA methylation, and is typically enriched in acetylation of lysine residues at histones H3 and H4 and in mono- and tri-methylation of H3-Lys4 (Table 1) [27, 28].

NcRNAs

Findings over the past ten years have progressively revealed the relevance of ncRNAs in most epigenetically controlled events including modulation of gene transcription, transposon activity and silencing, X-chromosome inactivation and paramutation [29]. NcRNAs include multiple classes of RNA transcripts that do not encode proteins but rather regulate gene expression at the post-transcriptional level

Table 1 Epigenetic modifications

Epigenetic modifications	Enzymes	Type of modification	Effect on gene expression	References
DNA Methylation	DNMT1 DNMT3A, DNMT3B	Methylation maintenance de novo methylation	Suppression Suppression	[17]
	TET family	De-methylation	Activation	[16]
Histone modifications	HATs	Acetylation	Activation e.g., H3K9, H3K14, H3K18, H3K56, H4K5, H4K8, H4K12, H4K16.	[25–28]
	HDACs	De-Acetylation	Repression	[25–28]
	HMTs	Methylation	Activation e.g., H3K4me2, H3K4me3, H3K36me3, H3K79me2 Repression e.g., H3K9me3, H3K27me3, H4K20me3.	[25–28]
	HDMs	De-methylation	Activation or repression based on the lysine residue	[25–28]
	MSK1	Phosphorilation	Activation e.g., H3S28	[26]
miRNAs		Non-perfect complementarity	Inhibition of the mRNA target translation	[35]
		Perfect complementarity	Degradation of the mRNA target	[35]

Overview of the epigenetic modifications and of the different classes of enzymes involved in these processes

DNMTs DNA methyltransferases, *TET* ten–eleven translocation enzymes family, *HATs* histone acetyltransferases, *HDACs* histone deacetylases, *HMTs* histone *N*-methyl transferases, *HDMs* histone demethylases, *MSK1* mitogen-and-stress activated protein kinase-1, *H3* histone 3, *H4* histone 4, *K* lysine residue, *S* serine residue, *Me2* di-methylation, *Me3* tri-methylation

[30]. The most extensively studied ncRNAs are the miRNAs, small RNA molecules (21–25 nucleotides in length) often implicated in cell- and tissue-specific differentiation and development and associated to different disorders [31]. In the human genome, the exact number of reported sequences coding for these regulatory molecules continues to rise. In 2015, an analysis of 13 human cell types has revealed the existence of 3707 novel miRNA sequences, in addition to the 1900 sequences previously described [32]. miRNAs in the human genome are transcribed from both introns and exons of non-coding genes and from introns of protein coding genes as well [33]. In addition, some mammalian miRNAs derived from various transposons and processed pseudogenes [34]. miRNAs are critical regulators of post-transcriptional gene expression. In particular, suppression of the target gene expression mediated by miRNAs occurs based on the degree of complementarity of the miRNA with the 3' Untranslated Region (3'UTR) of the target mRNA. Non-perfect complementarity between miRNA and target RNA, generally due to a pairing of only six to eight nucleotides, causes translation inhibition of the target mRNA, while near-perfect complementarity results in the degradation of the target mRNA by the RNA-induced silencing complex (Table 1) [35]. Interestingly, miRNAs are also susceptible to epigenetic modulation. Aberrant DNA methylation of miRNA gene promoters frequently occurs in human cancer and results in miRNAs expression down-regulation [36]. On the other hand, miRNAs are able to regulate both DNA methylation and histone modifications. Indeed, miRNAs may control the expression of important epigenetic regulators including DNMTs and HDACs thereby impacting on the entire gene expression profile [37].

Epigenetics in T2D and obesity

T2D and obesity are common metabolic disorders, which have reached epidemic proportions globally [38, 39]. Population and family (including twins) studies have extensively documented the familial aggregation of these diseases [40–45] with more than 175 genetic loci conclusively associated [46, 47]. Nevertheless, the impact of these loci, even in combination, on risk is very modest (5–10 % for T2D and ~2% for body mass index, BMI), leaving the heritability issue unsolved [48]. Technical limitations might, in part, account for this situation [49]. More likely, inheritance may be explained by epigenetics. Indeed, familial aggregation may reflect not only genetic influences, but also represent the effects of a shared family environment and thus of common environmentally induced epigenetic modifications [48]. In addition, while not been proved in humans yet, in rodents, certain environmentally induced epigenetic modifications can be trans-generationally transmitted to the offspring [50–52]. Environmentally induced epigenetic modifications may further explain the global epidemics of

Table 2 DNA methylation in T2D and Obesity

Genes	Regions	Epigenetic modification	Phenotypes	Tissues/cells	References
PGC1α	Promoter	↑ DNA Methylation	T2D	Pancreatic islets, Skeletal muscle	[58] [59]
PDX-1	Promoter	↑ DNA Methylation	T2D	Pancreatic islets	[63]
FTO	Intron 1	↓ DNA Methylation	T2D	PBLs	[64]
POMC	Intron 2-Exon 3	↑ DNA Methylation	Obesity	PBLs	[72]
RXRA	Promoter	↑ DNA Methylation	Obesity	Umbilical cord	[74]

Examples of epigenetically modulated genes in T2D and obesity

T2D type 2 diabetes, *PBLs* peripheral blood lymphocytes, *PGC1α* peroxisome proliferator activated receptor gamma coactivator-1 alpha, *PDX-1* pancreatic duodenal homeobox1, *FTO* fat mass and obesity-associated, *POMC* pre-proopiomelanocortin, *RXRA* retinoid X receptor-alpha

T2D and obesity, whose exponential rise in the past decades have been related to rapid cultural and social changes, such as socio-economic status, dietary changes, physical inactivity and unhealthy behaviors, all of which tend to cluster in family groups [38, 53]. Finally, epigenetics may help to understand the identical twin discordance for obesity and T2D [54, 55]. For example, the concordance rates for T2D among monozygotic twins are only ~ 70 % [56]. In these metabolic disorders, the incomplete concordance may be in part due to stochastic or environmentally determined epigenetic modifications that change over the lifetime and is responsible for the phenotypic differences and susceptibility to disease. Epigenetic processes may, therefore, contribute to the development of T2D and obesity and mediate the effects of environmental exposure on risk [9, 57]. In the following paragraphs, examples from our own as well as other investigators will be presented supporting the evidence linking epigenetic modifications, in particular DNA methylation, to T2D and obesity in both humans and rodents.

T2D and DNA methylation

T2D is a metabolic abnormality characterized by elevated plasma glucose levels. T2D typically occurs when insulin secretion fails to keep pace with reduced sensitivity to the action of circulating insulin [38]. There is now substantial evidence indicating that environmentally induced epigenetic changes contribute to diabetes prevalence (Table 2). Ling et al. have recently demonstrated that the promoter of the transcriptional co-activator *Peroxisome proliferator activated receptor gamma coactivator-1 alpha* (*PGC1-α*) gene, mainly involved in mitochondrial function, is highly methylated in pancreatic islets obtained from diabetic patients compared with non-diabetic controls [58]. Additionally, Barrès et al. have shown that the hyper-methylation of the *PGC-1α* promoter occurs even in the skeletal muscle from type 2 diabetic subjects compared with normal glucose-tolerant (NGT) individuals. Hyper-methylation negatively correlates with *PGC-1α* mRNA expression in these subjects [59]. In addition, the exposure of primary human skeletal muscle cells from NGT individuals to external factors, such as free fatty acids and tumor necrosis factor-alpha (TNF-α) directly and acutely alters the methylation status of *PGC-1α* promoter. These findings illustrate how alterations in the extracellular milieu may predispose to T2D by inducing DNA methylation changes [59]. Also, a genome-wide DNA methylation analysis of skeletal muscle from obese subjects before and after bariatric surgery provides evidence that the promoter methylation of *PGC-1α* is altered by obesity and restored after weight loss. DNA methylation inversely correlates to BMI, leptin, triglyceride and insulin levels in these subjects, which supports the role of DNA methylation in the physiological control of *PGC-1α* gene transcription [60]. The *Pancreatic duodenal homeobox 1* (*PDX-1*) promoter is also methylated. PDX-1 is a homeodomain-containing transcription factor that plays a key role in pancreas development and function. In humans, mutations of *PDX-1* cause maturity onset diabetes of the young 4 (MODY4) [61], while *Pdx-1* silencing in pancreatic β-cells causes diabetes in mice [62]. In humans pancreatic islets, Yang et al. have shown that 10 CpG sites in the *PDX-1* promoter and enhancer regions are hyper-methylated in type 2 diabetics compared with healthy individuals and that glycosylated hemoglobin (HbA1c) negatively correlates with mRNA expression of *PDX-1* and positively correlates with DNA methylation, suggesting a role of chronic hyperglycemia in the modulation of *PDX-1* expression through epigenetic events [63]. In support of this concept, these authors also found an increased DNA methylation of the *Pdx-1* gene in clonal rat β-cells exposed to high levels of glucose associated to increased mRNA expression and binding of the Dnmt1 on *Pdx-1* promoter [63]. More recently, a genome-wide analysis of differentially methylated sites in genomic regions associated to T2D has recently revealed that the *Fat mass and Obesity-associated*

(FTO) gene is hypo-methylated in a CpG site within the first intron in type 2 diabetics compared with control subjects in human peripheral blood. The T2D predictive power of this mark is significantly greater than all genetic variants so far described [64]. In the same investigation Toperoff et al. have also prospectively established, that, in an independent cohort hypo-methylation at the *FTO* intron is observed in young subjects that later progress to T2D. This further finding provides evidence that methylation changes predispose to T2D and deserve to be considered further investigated as T2D markers.

Obesity and DNA methylation

Obesity is a complex disorder resulting in an abnormal accumulation of fat in the organism due to alterations in energy homeostasis in terms of balance among energy intake, expenditure and storage [65]. It has been extensively documented in both humans and animal models that a relationship exists between obesity and the epigenetic regulation of genes involved in the control of food intake (Table 2) [51, 66–70]. In this context, the epigenetic modulation of the *Agouti* gene is a paradigmatic example of this association in mice. The *Agouti* gene encodes the paracrine-signaling molecule "Agouti signalling peptide" (ASIP), which antagonizes the melanocortin 1 receptor (MC1R) and leads melanocytes to produce yellow rather than black coat pigments. Additionally, ASIP acts as antagonist of the hypothalamic MC4R, inhibiting the anorexigenic neuropeptide alpha-melanocyte-stimulating hormone (α-MSH) signaling, thereby promoting the activation of orexigenic pathways which make mice hyperphagic and prone to develop obesity and diabetes [67]. It is now known that the *Agouti* gene is sensitive to cytosine methylation [69]. When its promoter is un-methylated, the *Agouti* gene is in an "ON" state, ASIP protein is abundant and mice show the typical agouti yellow coat and a tendency to develop obesity and diabetes [70]. On the contrary, when the promoter is heavily methylated, the *Agouti* gene is in an "OFF" state, ASIP levels are low resulting in mice that are lean and exhibit black coat. Interestingly, the *Agouti* gene is sensitive to environmental stimuli [51, 68, 71]. Nutrients and environmental pollutants impact on *Agouti* gene expression altering disease susceptibility through epigenetic modifications. *Agouti* pregnant mice fed diets supplemented with the methyl donors folic acid, vitamin B12 or choline generate lean brown offspring which show increased DNA methylation on the *Agouti* gene promoter and decreased ASIP protein levels [71]. In addition, the effects on coat color induced by maternal methyl-donor supplemented diet are also inherited in the F2 generation, indicating a germline propagation of the epigenetic modifications [51]. On the other hand, maternal exposure to the environmental pollutant bisphenol A, which is commonly present in many items such as food and plastic beverage containers and baby bottles, shifted the coat color of the offspring toward yellow by decreasing DNA methylation of CpG sites within the *Agouti* promoter [68]. Maternal supplementation with methyl donors abolished the bisphenol A-induced hypo-methylation of the *Agouti* gene in the offspring, demostrating the potential protective effect of simple dietary interventions against effect of an unhealthy environment effects on the fetal epigenome [68]. In humans, DNA methylation of the *Pre-proopiomelanocortin (POMC)* gene which encodes the anorexigenic hormone α-MSH produced by hypothalamic arcuate nucleus neurons has been associated with the individual risk of childhood obesity [72]. In particular, using peripheral blood cells, Kuenen et al. have found hyper-methylation at the Intron 2/Exon 3 boundary of the *POMC* gene in obese compared with normal weight children. In particular, in these obese children, the *Alu* elements, which are known to influence methylation in their genomic proximity at the Intron 2, trigger a default state methylation at the Intron 2/Exon 3 boundary, interfering with binding of the histone acetyltransferase/transcriptional coactivator p300 and reducing *POMC* expression [72]. In addition, several studies suggest a critical role of epigenetic marks also as predictors of susceptibility to obesity and metabolic disease in humans and animal models [73, 74]. A further example of this concept in humans has been provided by studies on the *Retinoid X receptor-alpha (RXRA)* gene. Godfrey et al., designed a perinatal epigenetic analysis of the methylation status of CpG sites at the promoters of 78 selected candidate genes in DNA from umbilical cord tissue of children who were assessed for adiposity at age 6 and 9 years. These authors have established that the variation of adiposity and the onset of obesity in pre-pubertal children were associated with the specific hyper-methylation of a CpG site at the RXRA chr9:1363558885+ at birth [74]. Furthermore, in the same population, it was demonstrated that this neonatal epigenetic mark was associated with lower maternal carbohydrate intake in pregnancy first trimester, providing a further example of how epigenetic processes may link the early prenatal life with the predisposition to obesity and other phenotypic outcomes [74]. In the future, perinatal identification of individuals that present DNA methylation changes at specific genes may help in preventing later obesity. In accordance to this concept, a recent bioinformatic analysis, performed to search for epigenetic obesity biomarkers, has established potential regions of interest which have a high density of CGIs in the promoter of several obesity-related genes (epiobesigenes), such as *Leptin*, *Phosphatase and tensin homolog* (*PTEN*), and *Fibroblast growth factor 2* (*FGF2*) or genes implicated in adipogenesis, such as *Peroxisome proliferator-activated receptor gamma* (*PPARG*), in inflammation, such as *Suppressors of*

cytokine signaling 1 and 3 (*SOCS1/SOCS3*), and insulin signaling, like *Lipoprotein lipase* (*LPL*), *Fatty acid binding protein 4* (*FABP4*), and *Insulin-like growth factor binding protein-3* (*IGFBP3*) [75].

Epigenetics and nutrition: lessons from recent studies

Nutritional epigenetics has become an attractive field of study since it associates nutrients and bioactive food components with epigenetic modifications of gene function. As reported in this review, a variety of evidence, in both humans and animal models, supports the association between changes in nutritional status, epigenetic modifications and predisposition to T2D and obesity [76]. Shen et al. have demonstrated that high fat diet (HFD) feeding impacts on the *Leptin* gene by inducing promoter CGI hyper-methylation in murine white adipose tissue (AT) [66]. Consistent with this observation, data obtained by our own group have further highlighted the role of over-nutrition in contributing to the gene function de-regulations occurring in obesity through epigenetic modification. By methylated DNA immuno-precipitation sequencing (MeDIP-seq), we have recently revealed that, HFD triggers a massive DNA methylation reprogramming in AT [77]. In particular, about 14.8×10^3 regions were found to be differentially methylated (DMRs) in mice fed a HFD. Interestingly, we have demonstrated that prolonged HFD regimen promotes a specific DMR distribution in mice [77]. DMR occurrence was increased in the gene-associated elements, particularly introns, and in the CGIs, while the number of DMRs identified in genomic repeat elements including long terminal repeats (LTRs) and long interspersed elements (LINEs) was decreased in obese compared with lean mice. Gene ontology analysis indicates that HFD feeding promotes DMRs enrichment in genes involved in developmental, metabolic and transcriptional processes in mice. In this same study, it has also been revealed that, among several differentially methylated pathways, the *Hox* family of transcription factors was highly enriched in differentially methylated genes in HFD-fed compared with STD-fed mice. In particular, the *Hoxa5* gene, which is implicated in fat tissue differentiation and remodeling [78, 79], was highly methylated at its 5′UTR and transcriptionally repressed in AT from obese compared with lean mice. In addition, the exposure of murine 3T3-L1 adipocytes to palmitate, a major component of the HFD, enhances methylation at the *Hoxa5* 5′UTR and causes *Hoxa5* mRNA down-regulation, suggesting that in AT the epigenetic silencing of *Hoxa5* gene may be dependent, at least in part, on the effect of saturated fats rather than on obesity per se [79].

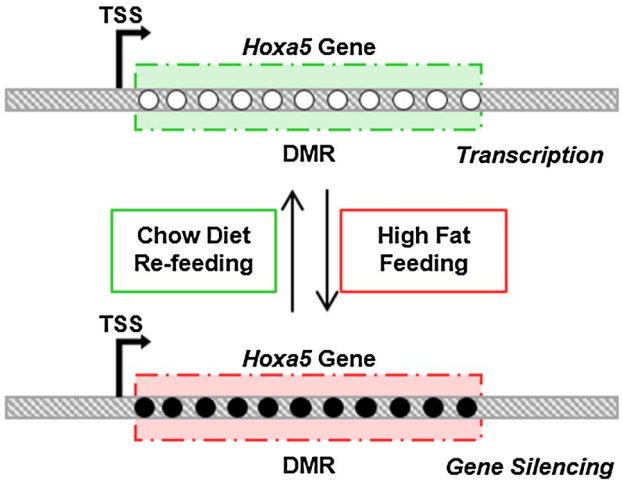

Fig. 2 High fat feeding: DNA methylation at the *Hoxa5* gene in vivo. DNA methylation analysis performed on the AT from mice fed chow diet or high fat diet reveals the presence of a DMR relative to the *Hoxa5* locus among high fat fed- and chow diet fed-mice. This event is associated with a strong reduction of the *Hoxa5* mRNA expression levels in obese mice compared with lean mice. Interestingly, re-feeding obese mice a chow diet for 2 further months reverted the *Hoxa5* DNA methylation and its expression to levels similar to those in lean control fed a standard chow diet

Interestingly, when obese mice exposed to chronic HFD treatment were returned to standard chow diet for two further months, *Hoxa5* DNA methylation and expression levels returned to values similar to those of mice maintained under STD, emphasizing the plasticity of these epigenetic events [79] (Fig. 2).

This same MeDIP-seq approach has also identified the *Ankyrin repeat domain 26* (*Ankrd26*) as a gene sensitive to nutrition-induced epigenetic changes. *ANKRD26*, a gene highly expressed in different areas of the hypothalamus, has been related to specific forms of hereditary obesity in humans [80] and was demonstrated to be involved in the regulation of feeding behavior and in the development of both obesity and diabetes in mice [81–83]. Mice with a partial inactivation of this gene show an obese phenotype which results from a marked hyperphagia rather than a reduction of the energy expenditure and activity [81]. When deleted at its C-terminus, Ankrd26 leads to excessive food intake and obesity due to severe region-specific changes in primary cilia in the brain [83]. In addition to its function in appetite control, the *Ankrd26* gene has a role in the regulation of adipocyte differentiation in mouse embryonic fibroblasts and in 3T3-L1 cells [84, 85]. We have demonstrated that hyper-methylation of the *Ankrd26* promoter occurs in obese mouse AT upon prolonged HFD feeding compared to age- and sex-matched STD-fed mice and directly interferes with the binding of the histone acetyltransferase/transcriptional coactivator p300 to

this same region. These events result in the down regulation of the *Ankrd26* gene expression [unpublished observations]. We have further reveled that *Ankrd26* silencing alters secretion of pro-inflammatory adipokines in vitro. These findings indicate that the epigenetic silencing of the *Ankrd26* gene might be one of the mechanisms responsible for AT inflammation in response to HFD [unpublished observations]. In humans, computational data from a genome-wide DNA methylation analysis in subcutaneous white AT revealed that *ANKRD26* gene is included in a list of 2825 genes where both DNA methylation and mRNA expression levels significantly correlate with BMI [86]. According to these findings, our preliminary data in human peripheral blood leukocytes underline a negative correlation between *ANKRD26* mRNA expression levels and BMI, supporting the hypothesis that an epigenetic regulation of *ANKRD26* gene may occur in humans, as well as in mice, and represent a pathogenic mechanism by which environmental exposures to nutrients contribute to disease susceptibility through epigenetic modifications.

Conclusions and future perspectives

The epigenome undergoes continuous transformations throughout our own lifetime leading to changes in genome function. The epigenetic hypothesis argues that, in addition to genetic variation, epigenetics provides an additional set of mechanisms mediating the relationship between genotype and the external environment and potentially contributing to the individual susceptibility to different disorders. During the past decades, the study of epigenetic modifications has been one of the most emerging and novel areas in fundamental as well as in clinical research and currently represents a very productive field of study, which has already provided a framework for the search of etiological factors in environment-associated diseases such as T2D and obesity. It is, indeed, clear that genetic variability only marginally contributes to the pathogenesis and family risk of these disorders. In this review, we have presented important acquisitions on the epigenetic network in T2D and obesity, mostly focusing on changes of the DNA methylation status of specific genes. However, understanding of the epigenetics of these two complex diseases is still limited. Further work is needed to clarify the molecular mechanisms responsible for the epigenetic control of gene activity and their interactions and alterations, and to establish the role of epigenetics in the risk stratification of these diseases. In the near future, further hints on how epigenetic changes are involved in the etiopathogenesis of T2D and obesity will be attained from studies accomplished on other epigenetic modifications, such as histone modifications and ncRNAs, which may selectively affect the expression of specific genes, and from epigenome-wide analysis extended to specific human cell types, e.g., stem, precursor and differentiated cells, or to particular tissues, e.g., fat, skeletal muscle, liver and pancreatic islets. It is expected that these studies will pave the way to novel and more effective strategies aimed at diabetes and obesity prevention and at personalized epigenetic treatment. Indeed, it becomes clear that getting a full picture of the epigenetic events involved in these two diseases will (1) represent a powerful tool for predicting and preventing future disease onset in the population; (2) provide additional stimuli for the development of clinical epigenetic biomarkers, which will generate novel relevant information for diagnosis, prognosis and therapy optimization; and (3) drive advancement in epigenetic drug discovery with the generation of more effective epi-drugs, selective for specific epi-targets, which in the forthcoming future might be used in combination with conventional therapeutics and/or might get the opportunity to develop epigenetic treatment personalized to the patient's epigenetic traits.

Compliance with ethical standards

Funding This study was funded by the European Foundation for the Study of Diabetes (EFSD), by the Ministero dell'Università e della Ricerca Scientifica (grants PRIN and FIRB-MERIT, and PON 01_02460) and by the Società Italiana di Diabetologia (SID-FO. DI.RI). This work was further supported by the P.O.R. Campania FSE 2007-2013, Project CREMe.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent For this type of study informed consent is not required.

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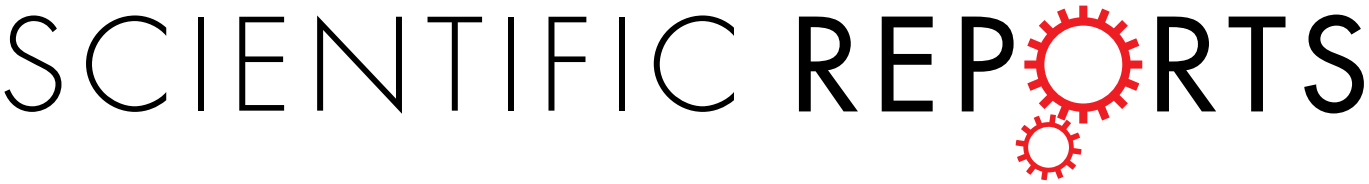

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Received: 02 August 2016
Accepted: 27 January 2017
Published: 07 March 2017

Specific CpG hyper-methylation leads to *Ankrd26* gene down-regulation in white adipose tissue of a mouse model of diet-induced obesity

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Epigenetic modifications alter transcriptional activity and contribute to the effects of environment on the individual risk of obesity and Type 2 Diabetes (T2D). Here, we have estimated the *in vivo* effect of a fat-enriched diet (HFD) on the expression and the epigenetic regulation of the *Ankyrin repeat domain 26* (*Ankrd26*) gene, which is associated with the onset of these disorders. In visceral adipose tissue (VAT), HFD exposure determined a specific hyper-methylation of *Ankrd26* promoter at the −436 and −431 bp CpG sites (CpGs) and impaired its expression. Methylation of these 2 CpGs impaired binding of the histone acetyltransferase/transcriptional coactivator p300 to this same region, causing hypo-acetylation of histone H4 at the *Ankrd26* promoter and loss of binding of RNA Pol II at the *Ankrd26* Transcription Start Site (TSS). In addition, HFD increased binding of DNA methyl-transferases (DNMTs) 3a and 3b and methyl-CpG-binding domain protein 2 (MBD2) to the *Ankrd26* promoter. More importantly, *Ankrd26* down-regulation enhanced secretion of pro-inflammatory mediators by 3T3-L1 adipocytes as well as in human sera. Thus, in mice, the exposure to HFD induces epigenetic silencing of the *Ankrd26* gene, which contributes to the adipose tissue inflammatory secretion profile induced by high-fat regimens.

Obesity and T2D are two common non-communicable diseases, which are now reaching epidemic proportions globally[1–3]. Epigenetic processes may contribute to the development of these disorders and mediate the effects of environmental exposure on risk of both diseases. Indeed, studies in humans and animal models support the association between changes in the nutritional status, epigenetic modifications and predisposition to obesity and T2D[4–7].

White adipose tissue (WAT) is a major endocrine tissue actively involved in the maintenance of the metabolic homeostasis in response to nutrition and other environmental clues through changes in fat storage, tissue expansion and adipokine secretion[8]. In disease states, the failure of compensatory response results in an impaired endocrine function which leads to insulin resistance and metabolic derangement[9]. In particular, fat stored in VAT strongly correlates with metabolic alterations and has been shown to be an independent risk factor for obesity-associated comorbidities[10–12].

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Variable	16 week-old		30 week-old	
	STD (n = 12)	HFD (n = 12)	STD (n = 12)	HFD (n = 12)
Body weight (g)	24.7 ± 2.1	34.4 ± 3.2[a]	26.4 ± 2.8	38.8 ± 3.3[b,c]
Fasting glucose (mmol/l)	5.9 ± 0.9	7.7 ± 1.7[a]	5.8 ± 1.2	9.1 ± 1.8[b]
GTT AUC (mmol/l 120 min^{-1})	725.7 ± 103.3	1290.7 ± 162.4[a]	653.6 ± 150.0	1344.2 ± 172.2[b]
ITT AUCi (mmol/l 120 min^{-1})	741.6 ± 127.0	355.7 ± 132.2[a]	681.0 ± 128.9	312.9 ± 117.6[b]

Table 1. Metabolic characteristics of HFD- and STD-fed mice. 8-week-old male C57BL/6 J mice were fed a high-fat diet (HFD) or a standard chow diet (STD) for 8 and 22 weeks. Body weight, fasting blood glucose, glucose tolerance test (GTT) Area Under the Curve (AUC) and insulin tolerance test (ITT) AUCi were reported. Data are mean ± SD of determinations. [a]$p < 0.001$, 16-week-old HFD *vs* 16-week-old STD; [b]$p < 0.001$, 30-week-old HFD *vs* 30-week-old STD; and [c]$p < 0.001$, 30-week-old HFD *vs* 16-week-old HFD.

Ankrd26 was recently identified as a gene involved in the regulation of the feeding behavior and in the development of both obesity and T2D in mice[13–15]. *ANKRD26* maps at chromosome 10p12 in humans, a region associated with certain forms of hereditary obesity[16]. In mice, *Ankrd26* gene is highly expressed in both the hypothalamus and WAT and its partial inactivation induces marked hyperphagia, severe obesity and diabetes *in vivo*[13,14]. In addition, *in vitro* evidence indicates *Ankrd26* as a regulator of adipogenesis[17,18]. A methylome analysis of mouse epididymal WAT (eAT)[19], the largest and easy accessible VAT depots in rodents[20,21], has identified promoter hyper-methylation of *Ankrd26* gene in HFD-fed compared to age- and sex-matched chow diet-fed mice, suggesting that *Ankrd26* gene is amenable to nutritionally-induced epigenetic modifications. In this study, we aimed at establishing whether and how HFD modulates *Ankrd26* gene expression in VAT *in vivo* through epigenetic processes.

Results

HFD affects body weight, glucose homeostasis and insulin sensitivity in mice. HFD-fed mice were heavier than standard chow diet (STD)-fed mice and reached a 50% increase of body weight compared with controls after 22 weeks of diet regimens (Table 1). These mice also exhibited increased fasting blood glucose levels, impaired glucose tolerance upon glucose loading and reduced insulin sensitivity after insulin injection compared with control mice (Supplementary Fig. S1).

HFD impairs *Ankrd26* expression in mice. To establish whether HFD modulates *in vivo Ankrd26* expression, mRNA and protein levels were measured in eAT. Treatment with HFD for 22 weeks led to a significant decrease in both *Ankrd26* mRNA ($p < 0.001$) and protein ($p < 0.01$) levels in obese mice compared with controls (Fig. 1a and b). Similarly, HFD lowered *Ankrd26* mRNA levels in mesenteric VAT (Supplementary Fig. S2). HFD treatment for 4 additional weeks did not elicit any further decrease in *Ankrd26* mRNA expression in the HFD-fed mice (34 week-old STD, *Ankrd26* mRNA: $2.29 \times 10^{-3} \pm 0.11 \times 10^{-3}$ AU; 34 week-old HFD, *Ankrd26* mRNA: $1.36 \times 10^{-3} \pm 0.19 \times 10^{-3}$ AU; $p < 0.001$). Differently from the long-term treatment, both *Ankrd26* mRNA and protein levels showed no differences between HFD- and STD-fed mice upon 8 weeks of diet regimens (Supplementary Fig. S3a and b). Next, the *Ankrd26* gene expression was assessed *in vitro* by exposing 3T3-L1 adipocytes to either palmitate or oleate, representing saturated and unsaturated fatty acid species, which are abundant in the HFD, or alternatively to leptin, whose levels raise through obesity development[22]. Quantitative real-time PCR (qPCR) analysis showed that palmitate, but not oleate or leptin, reduced *Ankrd26* expression by about 25% (Supplementary Fig. S4a and b), suggesting that, at least in part, excess of saturated fats accounts for HFD-induced *Ankrd26* gene repression.

HFD induces DNA methylation at the *Ankrd26* promoter in mice. To discover whether HFD induces DNA methylation changes at the *Ankrd26* promoter and 5′-untraslated region (5′ UTR), we performed Methylated DNA Immunoprecipitation (MeDIP) assay on pooled eAT genomic DNA from STD- and HFD-fed mice. This analysis revealed a 2-fold increase in DNA methylation at a segment of the promoter region (S1; −462 bp/−193 bp) in HFD-fed mice, while no DNA methylation enrichment was observed in a second segment (S2; −158 bp/+140 bp; Fig. 1c). Consistently, palmitate but not oleate or leptin, enhanced S1 DNA methylation at the *Ankrd26* promoter in 3T3-L1 adipocytes (Supplementary Fig. S4c and d), as showed by MeDIP assay. To further determine the specific HFD-induced DNA methylation profile occurring at 9 CpGs located at −436 and −221 bp from the *Ankrd26* TSS, we adopted bisulfite sequencing analysis. High CpG methylation density was detected in obese mice compared with controls in 2 close cytosine residues at −436 and −431 bp from the *Ankrd26* TSS (Fig. 1d). The combined percentage of methylation at these sites was inversely related to the amount of *Ankrd26* mRNA (Fig. 1e). In parallel with mRNA expression, mice fed HFD or STD for 8 weeks showed no difference in the *Ankrd26* DNA methylation state (Supplementary Fig. S3c). In addition, in 16 week-old and 30 week-old STD-fed mice, no difference in both *Ankrd26* mRNA levels (16 week-old STD, *Ankrd26* mRNA: $2.10 \times 10^{-3} \pm 0.20 \times 10^{-3}$ AU; 30 week-old STD, *Ankrd26* mRNA: $1.96 \times 10^{-3} \pm 0.23 \times 10^{-3}$ AU; $p = 0.126$) and DNA methylation (16 week-old STD, DNA methylation: 51.7 ± 2.9%; 30 week-old STD, DNA methylation: 51.3 ± 4.8%; $p = 0.900$) were observed. All together, these data indicate that the long-term exposure to calorie overload, rather than aging, affects eAT *Ankrd26* expression and DNA methylation in mice.

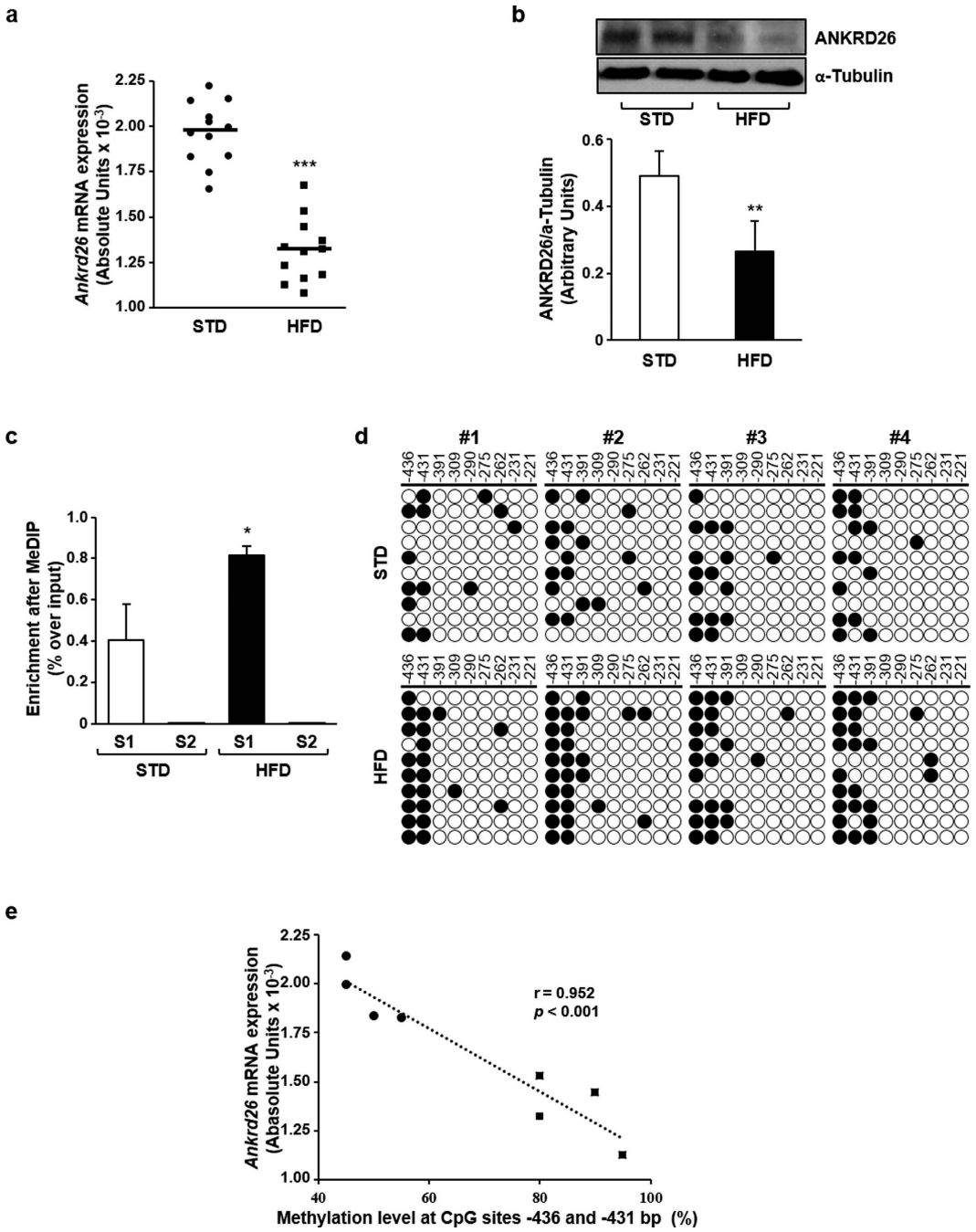

Figure 1. Ankrd26 expression and DNA methylation in eAT from mice upon 22 weeks of HFD or STD treatments. (**a**) qPCR of *Ankrd26* mRNA for HFD- (n = 12) and STD-fed (n = 12) mice. mRNA levels are expressed in absolute units (AU). (**b**) Representative western blot for ANKRD26 and α-Tubulin. Uncut western blot images are in the Supplementary Fig. S5. (**c**) MeDIP-qPCR of segment 1 (S1; −462 bp/−193 bp) and segment 2 (S2; −158 bp/+140 bp) of *Ankrd26* promoter region. (**d**) Bisulfite sequencing of *Ankrd26* promoter region (−436 bp/−221 bp) in HFD- (n = 4) and STD-fed (n = 4) mice. Each row indicates sequencing results of ten independent clones. White circles, un-methylated CpGs; black circles, methylated CpGs. CpG position relative to *Ankrd26* TSS is shown above each column. (**e**) Correlation between DNA methylation percentage at CpGs −436 and −431 bp and *Ankrd26* mRNA levels. *r* and *p* values are indicated on graph. (**b**,**c**) Results are mean ± SD from three independent experiments. (**a**–**c**), $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ *vs* STD.

Methylation at the CpGs −436 and −431 bp modulates *Ankrd26* promoter activity. To evaluate the causal relationship between the promoter DNA methylation and transcription of *Ankrd26* gene, a luciferase assay was performed in NIH-3T3 cells transfected with *in vitro* methylated (me) or un-methylated (unme) pCpG-*Ankrd26* luciferase reporter vectors, in which a selected region of the *Ankrd26* promoter was cloned,

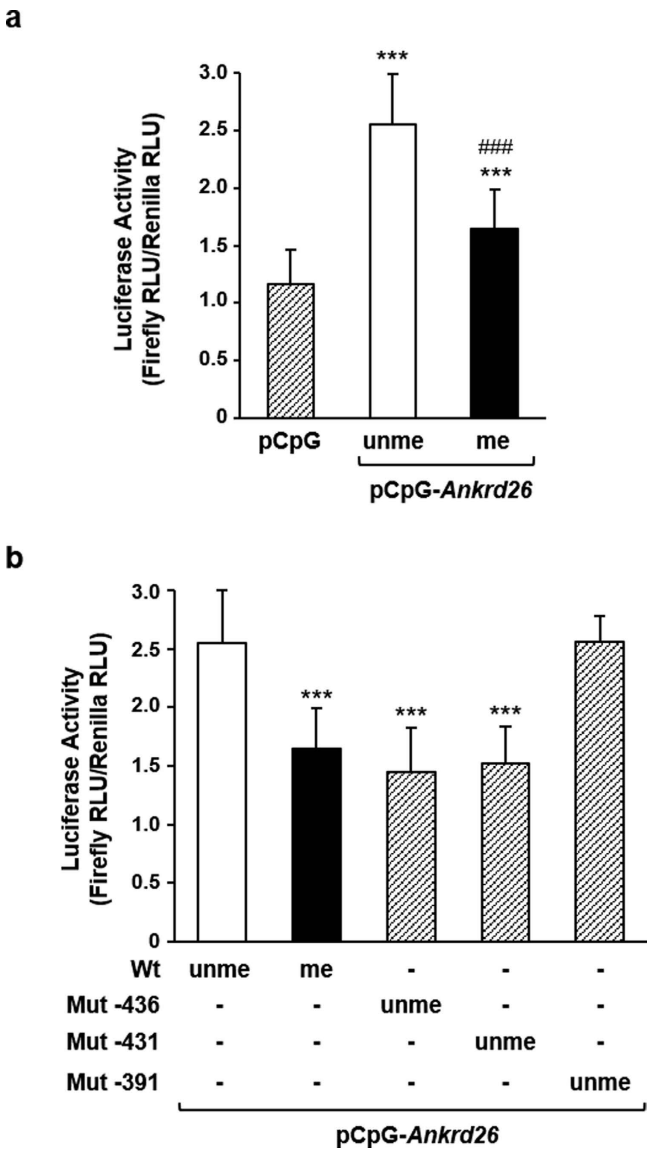

Figure 2. ***Ankrd26*** **promoter activity in NIH-3T3 cells.** (**a**) Luciferase activity of pCpG-*Ankrd26* constructs *in vitro* methylated (me) or un-methylated (unme) and of pCpG empty vector. Firefly luciferase activity was normalized to Renilla luciferase activity. Luciferase activity was measured in relative light units (RLU). $^{***}p < 0.001$ *vs* pCpG; $^{###}p < 0.001$ *vs* pCpG-*Ankrd26*unme. (**b**) Luciferase activity of unme mutagenized vectors, pCpG-*Ankrd26*-436, pCpG-*Ankrd26*-431 and pCpG-*Ankrd26*-391. Firefly luciferase activity was normalized to Renilla luciferase activity. Luciferase activity was measured in relative light units (RLU). $^{***}p < 0.001$ *vs* Wt unme. (**a**,**b**) results are mean ± SD from three independent experiments.

containing the CpGs −436, −431 and −391 bp. The un-methylated *Ankrd26* promoter induced a 2.5-fold increase in the luciferase activity compared with the empty vector (Fig. 2a), indicating that this selected fragment is sufficient to mediate promoter activity. In addition, methylation of the *Ankrd26* promoter caused a 35% decrease of luciferase activity compared with the un-methylated *Ankrd26* promoter (Fig. 2a), indicating that methylation of this region has a negative impact on *Ankrd26* gene expression. Next, to define whether the CpGs −436 or −431 bp or both is/are responsible for the regulation of the *Ankrd26* promoter activity, luciferase assays were performed in NIH-3T3 cells transfected with un-methylated site-specific mutagenized vectors. The un-methylated *Ankrd26* promoter mutagenized at the −436 bp CpG site (*Ankrd26*−436unme), similarly to the wild type (Wt) methylated *Ankrd26* promoter, showed a 40% reduction of the luciferase activity compared with the un-methylated *Ankrd26* Wt fragment (Fig. 2b). Similar data were obtained when the *Ankrd26* promoter was mutagenized at the −431 bp CpG site (Fig. 2b). Conversely, when the *Ankrd26* promoter was mutagenized at the −391 bp CpG site, the luciferase activity of the un-methylated *Ankrd26*−391 promoter was comparable to the un-methylated Wt promoter (Fig. 2b). Thus, specific methylation at the −436 and −431 bp CpGs in the *Ankrd26* promoter modulates *Ankrd26* gene expression *in vitro*.

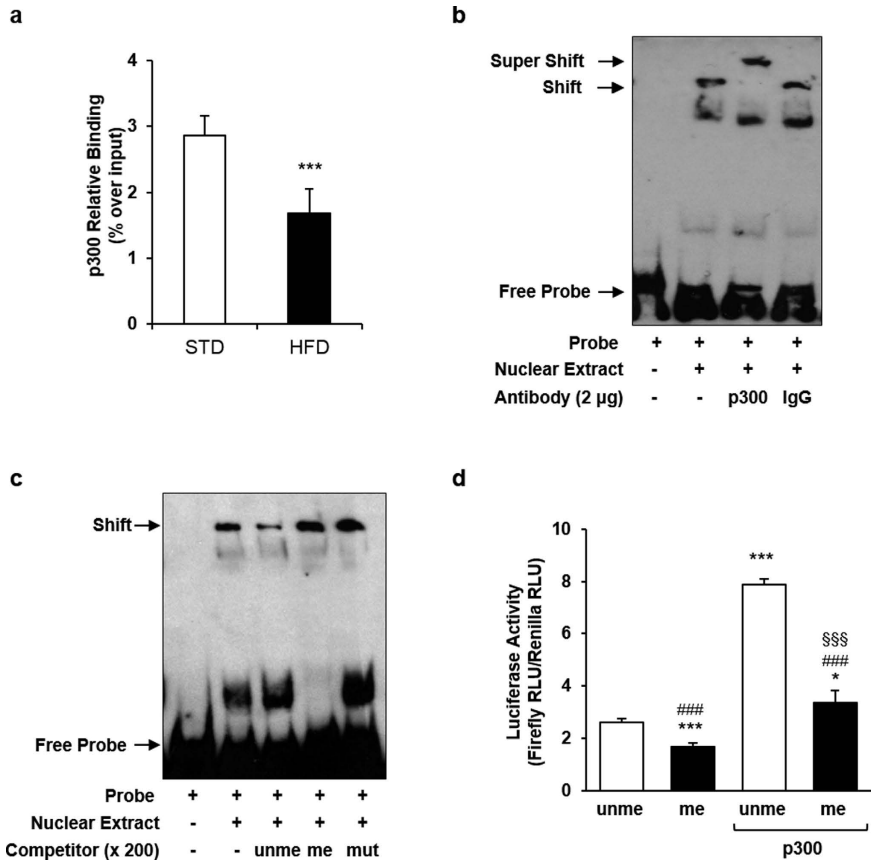

Figure 3. p300 binding and activity on *Ankrd26* promoter. (**a**) ChIP of p300 binding on *Ankrd26* promoter in eAT from HFD- (n = 3) and STD-fed (n = 3) mice, upon 22 weeks of diet regimens. ChIP enrichment is relative to Input chromatin. $^{***}p < 0.001$ *vs* STD. (**b**,**c**) Representative EMSA for double-stranded biotinylated *Ankrd26* probe with Nuclear Extract (NE) from NIH-3T3 cells. Uncut gel images are in the Supplementary Fig. S5. (**b**) EMSA super-shift assay with an anti-p300 antibody (lane 3) or a rabbit IgG (lane 4). (**c**) EMSA competition assay with 200-fold molar excess of un-labeled un-methylated (unme; lane 3), methylated (me; lane 4) or mutagenized (mut; lane 5) competitor. (**d**) Luciferase activity of *in vitro* methylated (me) or un-methylated (unme) pCpG-*Ankrd26* vector in NIH-3T3 cells co-transfected with pCl.*p300* vector. Firefly luciferase activity was normalized to Renilla luciferase activity. Luciferase activity was measured in relative light units (RLU). $^{***}p < 0.001$ *vs* pCpG-*Ankrd26* unme; $^{###}p < 0.001$ *vs* pCpG-*Ankrd26* unme + pCl.*p300*; $^{§§§}p < 0.001$ *vs* pCpG-*Ankrd26* me. (**a** and **d**), results are mean ± SD from three independent experiments.

Methylation at the CpGs −436 and −431 bp impairs p300 binding to the *Ankrd26* promoter. DNA methylation often induces gene silencing by inhibiting transcriptional activator binding to promoters[23]. TFBIND analysis of the *Ankrd26* promoter region spanning the CpGs −436 and −431 bp, predicted a consensus sequence (−442 bp/−429 bp) for the histone acetyltransferase/transcriptional coactivator p300[24,25]. The recruitment of p300 to this putative binding site and its relevance to the regulation of the *Ankrd26* gene expression was therefore investigated. Chromatin Immunoprecipitation (ChIP) analysis showed a 40% decrease in p300 binding to the *Ankrd26* promoter in eAT of obese compared with lean mice (Fig. 3a). In addition, Electrophoretic Mobility Shift Assay (EMSA) with a double-stranded labeled probe containing the p300 consensus sequence on the *Ankrd26* promoter revealed that the addition of p300 antibody to the probe/Nuclear Extract (NE) mix super-shifted one of the complexes formed by interaction of the probe with the NE proteins (Fig. 3b). Also, the presence of an un-methylated competitor to the probe/NE mix effectively displaced p300 binding to the probe, while the probe/p300 complex was not affected by the addition of both a methylated or a mutagenized competitor (Fig. 3c). The *in vitro* over-expression of p300 in NIH-3T3 cells caused a 3-fold increase of the un-methylated *Ankrd26* promoter activity (Fig. 3d). At variance, when p300 was over-expressed, the luciferase activity of the methylated *Ankrd26* promoter was 60% lower compared with the un-methylated *Ankrd26* promoter (Fig. 3d). All together, these data indicate that p300 binding to *Ankrd26* promoter regulates *Ankrd26* gene expression and is dependent on the methylation state of the CpGs −436 and −431 bp.

HFD induces hyper-methylation of the *Ankrd26* promoter through DNMT3a and DNMT3b in mice. The molecular events upstream and downstream methylation-induced displacement of p300 binding to the *Ankrd26* promoter were subsequently analyzed. ChIP analysis revealed that binding of DNMT3a and DNMT3b, but not of DNMT1, to the *Ankrd26* promoter was increased in HFD-fed mice compared to controls

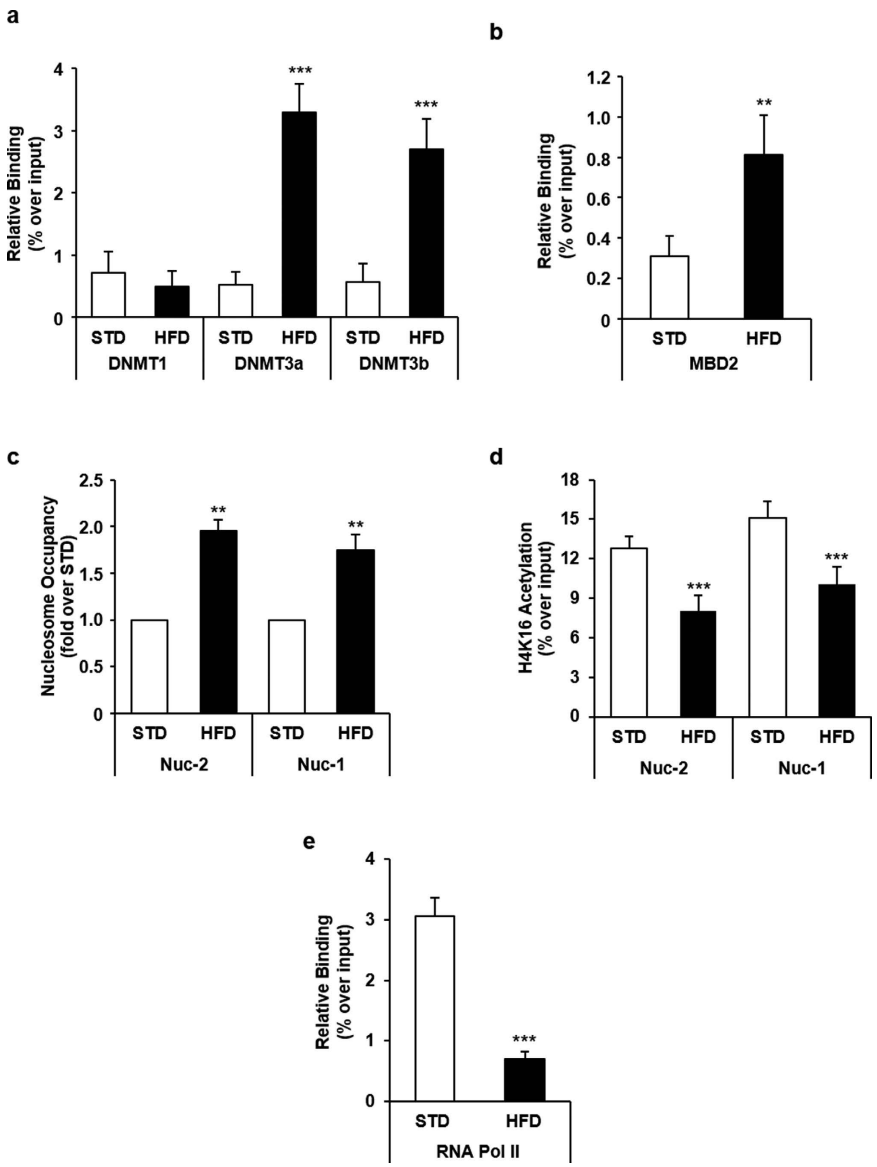

Figure 4. Epigenetic changes and protein binding at *Ankrd26* promoter in eAT from mice upon 22 weeks of HFD. ChIP of DNMT1, DNMT3a, DNMT3b (**a**) and MBD2 (**b**) binding at *Ankrd26* promoter region (−553 bp/−348 bp). (**c**) MNase for Nuc-2 (−257 bp/−198 bp) and Nuc-1 (−84 bp/−25 bp) occupancy at *Ankrd26* promoter. (**d**) ChIP for acetyl-H4 enrichment at Nuc-2 and Nuc-1. (**e**) ChIP of RNA Pol II binding at *Ankrd26* TSS (+16 bp/+159 bp). (**a**,**b**) and (**d**,**e**), ChIP enrichment is relative to Input chromatin. (**a**–**e**), results are mean ± SD from three independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs* STD.

(Fig. 4a). Interestingly, HFD increased the binding of methylation-dependent transcriptional repressor MBD2 as well (Fig. 4b)[23].

HFD affects histone acetylation, nucleosome positioning and RNA Pol II binding at the *Ankrd26* promoter in mice. Further analysis of the *Ankrd26* promoter using the NuPoP software predicted 2 nucleosomes (Nuc), Nuc-2 (−288 bp/−132 bp) and Nuc-1 (−105 bp/ + 41 bp), positioned between the p300 consensus sequence and the *Ankrd26* TSS. Micrococcal Nuclease (MNase) treatment of eAT chromatin from HFD- and STD-fed mice followed by qPCR revealed that HFD rendered the *Ankrd26* promoter less sensitive to nuclease digestion, increasing Nuc-2 and Nuc-1 positioning (Fig. 4c). Consistently, ChIP analysis showed that HFD feeding significantly lowered histone H4 acetylation at both nucleosomes in the HFD- compared to STD-fed mice ($p < 0.001$; Fig. 4d). The RNA Pol II binding to the *Ankrd26* TSS was also significantly lower in HFD-fed mice ($p < 0.001$; Fig. 4e). All together, these data indicate that methylation at the CpGs −436 and −431 bp and the subsequent p300 displacement from this region silenced *Ankrd26* expression through nucleosome remodeling at the *Ankrd26* promoter.

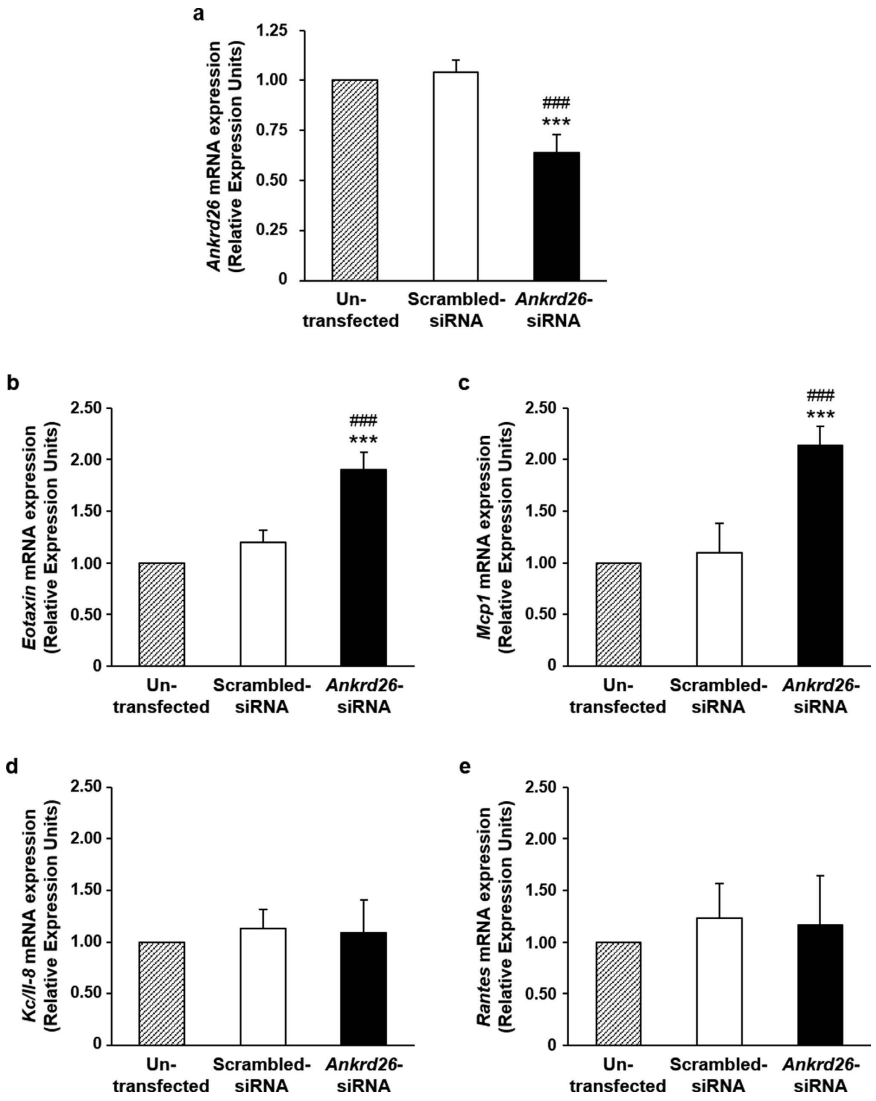

Figure 5. *Ankrd26* mRNA expression in *Ankrd26*-silenced mature adipocytes. 3T3-L1 mature adipocytes were silenced with 25 nmol/l of scrambled-siRNA or *Ankrd26*-siRNA for 48 h. Un-transfected cells were used to exclude transfection interference on mRNA expression. *Ankrd26* (**a**), *Eotaxin* (**b**), *Mcp1* (**c**), *Kc/Il-8* (**d**) and *Rantes* (**e**) mRNA levels were evaluated at the end of the experiment and expressed in Relative Expression Units (REU). Data are mean ± SD of determinations from three independent experiments. $^{***}p < 0.001$, *vs* Un-trasfected; $^{###}p < 0.001$, *vs* Scrambled-siRNA.

***Ankrd26* silencing promotes secretion of pro-inflammatory chemokines by cultured adipocytes.** To assess the functional consequences of the HFD-induced epigenetic silencing of the *Ankrd26* gene, *Ankrd26* mRNA was reduced by about 35% by transfecting *Ankrd26*-specific siRNA in 3T3-L1 adipocytes (Fig. 5a). Silenced adipocytes showed enhanced secretion of the pro-inflammatory chemokines, Keratinocyte-derived Cytokine/Interleukine 8 (KC/IL-8), Eotaxin, Monocyte chemotactic protein 1 (MCP1) and Rantes (Table 2). These changes were accompanied by increased mRNA levels of *Eotaxin* and *Mcp1* with no change in *Kc/Il-8* and *Rantes* mRNAs (Fig. 5b,c,d and e). It appeared therefore that Ankrd26 physiologically controls the adipocyte pro-inflammatory secretion profile through effects occurring at different levels.

***ANKRD26* expression negatively correlates with Body Mass Index (BMI) and inflammation markers in humans.** mRNA expression of *ANKRD26* in VAT was further examined in human obese subjects (Supplementary Table S1) in relation to BMI and inflammatory parameters. Interestingly, in obese subjects with normal glucose tolerance (NGT), *ANKRD26* expression in VAT was found to negatively correlate with BMI (Fig. 6a), with serum levels of the pro-inflammatory chemokines, IL-8 and RANTES and with serum levels of the inflammatory markers, IL-6 and C-reactive protein (CRP) (Fig. 6b,c,d and e). Altogether, these data indicate that, in obese humans, the reduction of *ANKRD26* gene expression is associated with increased body weight and with a pro-inflammatory status.

Variable	3T3-L1 Adipocytes		
	Un-transfected	Scrambled-siRNA	*Ankrd26*-siRNA
Eotaxin (pg/ml)	514.34 ± 73.25	521.16 ± 82.90	705.92 ± 98.99[b,d]
G-CSF (pg/ml)	6.45 ± 0.96	7.11 ± 0.22	7.14 ± 0.90
IL-4 (pg/ml)	3.41 ± 0.32	3.21 ± 0.44	3.81 ± 0.51
IL-5 (pg/ml)	0.88 ± 0.14	0.79 ± 0.16	0.83 ± 0.33
KC/IL-8 (pg/ml)	565.39 ± 15.32	588.67 ± 30.86	702.58 ± 34.08[a,c]
IL-17 (pg/ml)	1.44 ± 0.62	1.72 ± 0.51	1.56 ± 0.42
MCP1 (pg/ml)	1758.04 ± 72.31	1718.77 ± 248.29	2500.31 ± 225.38[b,d]
MIP1β (pg/ml)	1.32 ± 0.75	1.14 ± 0.52	1.17 ± 0.37
Rantes (pg/ml)	27.95 ± 2.74	33.31 ± 8.88	53.45 ± 2.35[b,d]
TNFα (pg/ml)	4.98 ± 1.46	5.29 ± 1.31	5.57 ± 1.81

Table 2. Effect of *Ankrd26* gene silencing on adipocyte-released chemokines/cytokines. 3T3-L1 mature adipocytes were silenced with 25 nmol/l of scrambled-siRNA or *Ankrd26*-siRNA for 48 h. Conditioned media were collected for 24 h in Dulbecco's modified Eagle's medium without serum and with 0.5% BSA. Adipokines were then assayed using the Bio-Plex Pro Mouse Cytokine Immunoassay. Un-transfected cells were also used to exclude transfection interference on adipokine secretion. Detectable adipokines are reported. Data are mean ± SD of determinations from three independent experiments. [a]$p < 0.001$ and [b]$p < 0.01$, *vs* Un-transfected; [c]$p < 0.001$ and [d]$p < 0.01$, *vs* Scrambled-siRNA. Granulocyte-colony stimulating factor, G-CSF; Interleukin, IL; Keratinocyte-derived Cytokine/Interleukine 8, KC/IL-8; Monocyte chemotactic protein 1, MCP1; Macrophage inflammatory protein 1 beta, MIP1β; Tumor necrosis factor alpha, TNFα.

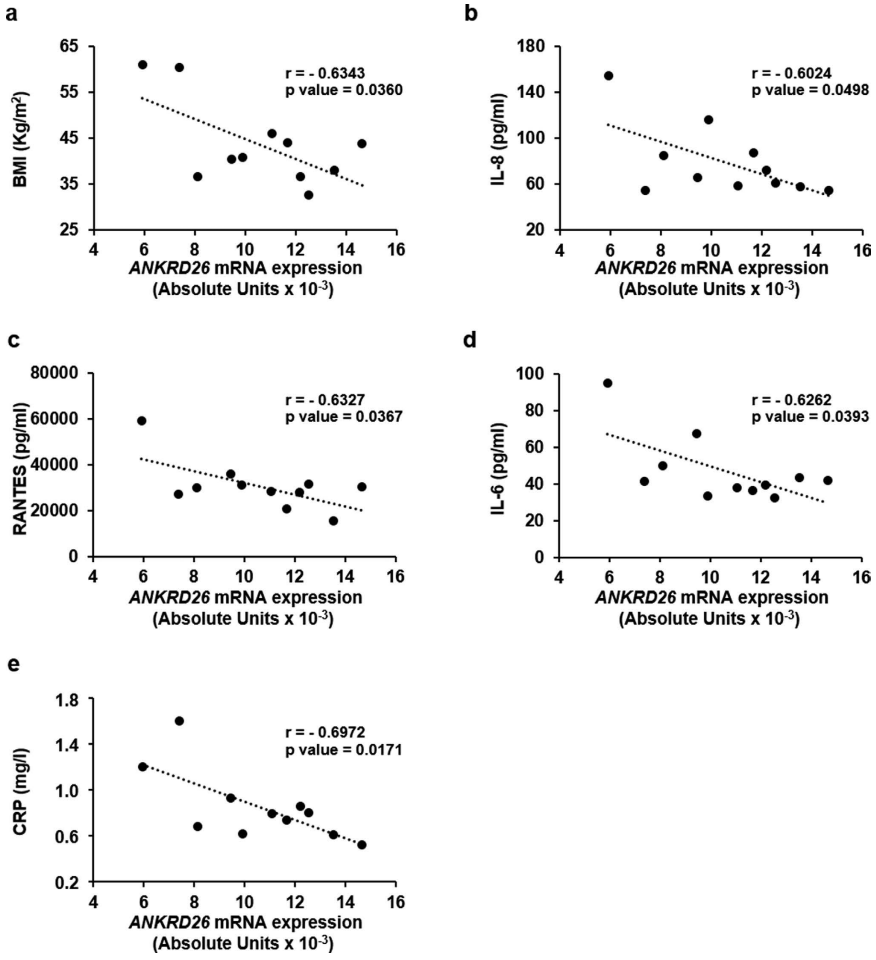

Figure 6. Associations between VAT *ANKRD26* mRNA expression, BMI and systemic inflammatory parameters in humans. Correlations of the human VAT *ANKRD26* mRNA expression with BMI (**a**) and with serum inflammatory markers, IL-8 (**b**), RANTES (**c**), IL-6 (**d**) and CRP (**e**) in normal glucose tolerant obese individuals (n = 11; 5 M/6 F). *r*, Pearson's coefficient; $p < 0.05$ were considered statistically significant.

Discussion

Epigenetic modifications represent a common mechanism through which both genetic and environmental exposures impact on the susceptibility to obesity and T2D[26–28]. Recent evidence has underlined the potential importance of epigenetic regulation of gene expression and function in obesity[26]. Methylation changes at the promoter of several genes have been identified in both human and rodent obesity[29]. Additionally, the exposure to high calorie diets, which promotes DNMT expression and enzymatic activities, impacts on DNA methylation profiles both at specific genes and at genome-wide level[22,30], suggesting that DNA methylation changes play a role in the responses to fat and high-calorie diets[31].

Our study revealed HFD-induced methylation of the *Ankrd26* promoter. We have no clue at the moment on the detailed mechanisms causing the increased methylation of *Ankrd26* in HFD-fed mice, but our results indicate that the HFD-induced hyper-methylation at *Ankrd26* promoter was concomitant with enhanced binding of *de novo* DNMT3a and DNMT3b to the same *Ankrd26* promoter region. These changes were followed by down-regulation of *Ankrd26* expression in the eAT. The epigenetic silencing of *Ankrd26* gene in eAT appear to depend, at least in part, on saturated fats, abundant in the HFD. Indeed, we found increased promoter DNA methylation and down-regulation of *Ankrd26* in 3T3-L1 adipocytes upon exposure to palmitate, a major component of the HFD, but not to oleate. At the variance with palmitate, cell exposure to leptin, whose serum concentration increases in relation to obesity[22], showed no effect on *Ankrd26* expression and methylation. These findings suggest that specific nutritional components of the HFD may contribute to the epigenetic silencing of *Ankrd26* gene.

The HFD-induced changes in DNA methylation at *Ankrd26* promoter and gene expression result from a long-term diet exposure. Indeed, cytosine hyper-methylation at the *Ankrd26* promoter and gene silencing appeared in eAT from obese mice after a prolonged HFD feeding, while no evident difference was observed at earliest time-point. This time effect was associated with the eAT compensatory remodeling occurring in response to HFD. Indeed, eAT, along with other VAT depots, contributes to the inflammatory and metabolic complications in murine obesity[32], and responds to HFD through different time-dependent changes[33,34]. Early upon HFD exposure (8–12 weeks), eAT expansion is accompanied by a major increase in adipocyte size. Upon more prolonged HFD exposure (20 weeks), however, eAT expansion is mainly sustained by increased adipogenesis and accompanied by enhanced secretion of inflammatory mediators, including Tumor necrosis factor alpha (TNF-α), IL-6, MCP1 and Rantes[33–35]. The mechanisms triggering this compensatory response in eAT have not been clarified yet but the present work now shows that they may involve HFD-induced *Ankrd26* down-regulation. Along with its role in feeding behavior and body fat accumulation[13–15], *Ankrd26* has been identified as a regulator of adipogenesis *in vitro*[17,18]. Firstly, adipogenesis of 3T3-L1 cells is enhanced by selective silencing of the *Ankrd26* gene with an *Ankrd26*-specific shRNA[18]. Secondly, Mouse Embryonic Fibroblasts from *Ankrd26* mutant mice (MEFs *Ankrd26*$^{-/-}$) have a higher rate of adipocyte differentiation. Indeed, the mRNA expression of the master regulator genes of differentiation process, *CCAAT enhancer-binding protein* α (*C/ebp*α), and *Peroxisome proliferator-activated receptor* γ (*Ppar*γ), are up-regulated in MEFs *Ankrd26*$^{-/-}$, indicating that this gene is involved in regulating both the pre-adipocyte commitment and differentiation[17].

In this work, we further demonstrated enhanced expression and/or secretion of the pro-inflammatory chemokines Eotaxin, MCP1, KC/IL-8, and Rantes by 3T3-L1 adipocytes whose *Ankrd26* expression was silenced to levels similar to those occurring in response to HFD. These cytokines have been reported to contribute to adipose tissue inflammation[36,37]. Since secretion of Eotaxin, MCP1, KC/IL-8, and Rantes by the eAT also increases upon prolonged exposure to HFD[33,34], our findings suggest the involvement of *Ankrd26* down-regulation in raising and/or sustaining the low-grade inflammatory response which occursin the eAT after long-termHFD feeding and is implicated in the development of insulin resistance and T2D[33–35]. This might represent a mechanism by which environmental cues are integrated at specific genomic loci, contributing to the metabolic disorder. The relevance of these observations to humans is supported by our further findings in obese individuals with normal glucose tolerance, revealing that the reduction of *ANKRD26* expression in VAT negatively correlates with the serum concentrations of inflammatory markers and pro-inflammatory chemokines, which are associated to obesity in humans[38,39] and whose increased levels predict occurrence of T2D[38–43]. Cardamone *et al.* have recently shown in the adipose tissue the relevance of the cytosolic function of the Ankrd26 partner GPS2 (G protein pathway suppressor 2) to the prevention of uncontrolled activation of inflammatory programs[44]. Even though this issue deserves further mechanistic investigation, we suggest that Ankrd26 might work as a molecular regulator of inflammatory signaling pathways, at least in part, by facilitating the cytoplasmic localization of its interacting partner GPS2[18]. Therefore, *ANKRD26* down-regulation might represent an early event triggering chronic low-grade inflammatory response in human adipose tissue.

Detailed methylation analysis of the *Ankrd26* promoter showed that HFD induced specific methylations at −436 and −431 bp CpGs, thereby exerting a suppressive effect on *Ankrd26* promoter activity. Similar to DNA methylation, mutagenesis at the *Ankrd26* promoter showed that introduction of C → T mutation at −436 or −431 bp CpGs significantly reduces *Ankrd26* promoter activity. These results provide evidence that *i.*, these cytosine residues have functional significance to the *Ankrd26* gene expression; and *ii.*, the DNA methylation at these specific CpGs plays a functional role in the epigenetic repression of *Ankrd26* gene.

Current evidence supports a role for epigenetic changes in the regulation of metabolic diseases and in some cases, as in our study, it has been demonstrated that small methylation changes are associated with gene expression variability with significant effects on the phenotype[45–48]. In support of this concept, Barrès *et al.*[46] have recently shown that hyper-methylation of the *Peroxisome proliferator-activated receptor* γ *coativator 1* α (*PGC1*α) promoter modulates *PGC1*α expression, implying a mechanism for decreased mitochondrial content in skeletal muscle from T2D patients. Also, using a gene reporter assay, these same authors have demonstrated that the *in vitro* methylation of a single cytosine residue at the *PGC1*α promoter is responsible for the reduction of gene activity[46].

CpG methylation generally affects transcription directly, by blocking the binding of transcriptional activators[24,49], or indirectly, by recruiting DNA-binding proteins and co-repressor complexes that occupy the methylated promoters and facilitate the formation of heterochromatin[50]. In this study, we have further demonstrated that *i.*, *in vitro*, the histone acetyltransferase/transcriptional coactivator p300 directly binds the consensus sequence at the *Ankrd26* promoter, containing the methylation sensitive cytosines −436 and −431 bp; *ii.*, hyper-methylation of these sites affects p300 binding and activity *in vivo* and *in vitro.* p300 regulates gene expression by acetylating both histones and transcriptional factors and plays a key role in modulating chromatin structure and function[25,26]. In this paper, we have also reported that HFD reduces histone H4 acetylation, increases nucleosome occupancy at the *Ankrd26* promoter, and impairs RNA Pol II binding to the *Ankrd26* TSS, suggesting that the HFD-dependent p300 displacement from the *Ankrd26* promoter silences *Ankrd26* gene. These findings are consistent with recent studies demonstrating that CpG methylation suppresses transcription of several genes by direct inhibition of p300 binding to their promoter sequences[51–53]. In conjunction with the inhibition of p300 binding, HFD induced the binding of MBD2 to the *Ankrd26* promoter in mice. MBD2 is a methyl-CpG binding protein and causes gene silencing by recruiting histone deacetylase at the methylated promoter regions[31,50]. Accordingly, we propose that the specific CpG methylation at the *Ankrd26* promoter leads to HFD-induced epigenetic gene silencing by triggering a cascade of events which involves DNA-associated regulatory proteins, such as p300 and MBD2, and changes in chromatin structure.

The potential relevance to humans of the findings reported in the present work is supported by our further evidence that VAT *ANKRD26* mRNA levels were negatively correlated with BMI in humans. Consistent with our results, very recent computational data from a genome-wide DNA methylation analysis in human adipose tissue have revealed that *ANKRD26* DNA methylation and mRNA expression correlate with BMI[53]. Thus, epigenetic regulation of *ANKRD26* gene may occur in humans as well.

In conclusion, our work reveals that the methylation of specific CpGs at the *Ankrd26* promoter occurs in mice during HFD treatment and causes the down-regulation of *Ankrd26* expression, at least in part, by impairing p300 binding to its promoter. We propose that the epigenetic silencing of the *Ankrd26* gene contributes to VAT inflammation following unhealthy dieting.

Methods

Animals, diets and tests. Animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication no. 85–23, revised 1996). Protocols were approved by the ethics committee of the "Federico II" University of Naples. Six-week-old C57BL/6 J male mice (n = 48) from Charles River Laboratories International, Inc. (Wilmington, MA) were housed in a temperature-controlled (22 °C) room with a 12 h light/dark cycle. Two weeks after arrival, mice were randomly divided into two groups of 12 mice each and were fed either a HFD (60 kcal% fat content; Research Diets formulas D12331; Research Diets, Inc., New Brunswick, NJ) or a standard chow diet (STD; 11 kcal% fat content; Research Diets formulas D12329; Research Diets, Inc.) for 8 and 22 weeks. The composition of these diets is reported in Supplementary Table S2. Body weight was recorded weekly throughout the study. The glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed as described[14,16]. Blood glucose levels were measured using a glucometer (One Touch Lifescan, Milan, Italy). Mice were killed by cervical dislocation. eAT was collected from each mouse, snap frozen in liquid nitrogen and stored at −80 °C.

Quantitative real-time PCR (qPCR) and western blot analysis. Tissues were homogenized by TissueLyser LT (Qiagen, Hilden, Germany) following manufacturer's protocol. RNA and DNA were isolated using AllPrep DNA/RNA/miRNA Universal kit (Qiagen). cDNA synthesis and qPCR were performed as described[19,54]. Immunoblotting was carried out as indicated[14]. Antibodies against ANKRD26 (#SC-82505, Santa Cruz Biotechnology, Inc., Dallas, TX), and α-Tubulin (#MA1-19162, Sigma-Aldrich, St. Louis, MO) were used for protein detection.

Methylated DNA Immuoprecipitation (MeDIP). MeDIP assay was performed as described[19]. DNA methylation enrichment was evaluated on genomic DNA isolated from eAT of STD- and HFD-fed mice and from 3T3-L1 adipocytes. Sonicated pooled genomic DNA from eAT or cells was immuno-precipitated using anti-5meCpG (#ab10805, Abcam, Cambridge, MA) or mouse IgG (#I8765, Sigma-Aldrich) with anti-mouse IgG beads (Life Technologies, Carlsbad, CA). DNA methylation enrichment on recovered DNA was evaluated by qPCR. Samples were normalized to their respective input using the $2^{-\Delta CT}$ method.

Bisulfite sequencing. For bisulfite sequencing analysis, we used genomic DNA isolated from eAT of STD- and HFD-fed mice. Bisulfite conversion of DNA was performed with the EZ DNA Methylation Kit (Zymo Research, Orange, CA), following manufacturer's instructions. Converted DNA was amplified by PCR. PCR products were cloned into the pGEM T-Easy vector (Promega, Madison, WI) and 10 clones for sample were sequenced on AB 3500 genetic analyzer (Life Technologies). DNA methylation percentage at the −436 and −431 bp CpGs for each mouse was calculated using the formula: DNA methylation % = [methylated CpGs/(methylated CpGs + unmethylated CpGs)]*100.

Cloning strategy, site-direct mutagenesis and *in vitro* methylation. *Ankrd26* promoter (−733 bp/−344 bp) was amplified by PCR. The purified PCR fragment was cloned into the firefly luciferase reporter pCpGfree-promoter-Lucia vector (Invivogen, Toulouse, France). The following site-specific mutated constructs were generated by PCR-based mutagenesis: pCpG-*Ankrd26*-436, pCpG-*Ankrd26*-431, pCpG-*Ankrd26*-391. The wilde type (Wt) pCpG-*Ankrd26* vector, used as template, was removed from the PCR reaction by *DpnI* digestion (New England BioLabs, Ipswich, MA). Wt and mutated (mut) vectors were amplified into *E. coli* GT115 cells (Invivogen). Site-specific mutagenesis of each construct was validated by sequencing. *In vitro* methylation

was performed using the *M.SsI* CpG methyltransferase following manufacturer's protocol (New England BioLabs). Un-methylated DNA was obtained in the absence of *M.SsI*. Methylation was confirmed by resistance to *HpyCH4IV* digestion (New England BioLabs).

Luciferase assay. NIH-3T3 cells were transfected with methylated or un-methylated Wt or mutagenized pCpG-*Ankrd26* vector and Renilla control vector (Promega) by lipofectamine (Life Technologies), following manufacturer's instructions. Where indicated, cells were co-transfected with pCl.*p300* expression vector (Promega). Firefly luciferase activity of each transfection was normalized for transfection efficiency against Renilla luciferase activity.

Chromatin Immunoprecipitation (ChIP) and Micrococcal Nuclease (MNase) assays. ChIP and MNase assays were performed as described[55,56]. Briefly, 100 mg of eAT were cross-linked with 1% formaldehyde for 15 min at 37 °C. For ChIP assay, sonicated chromatin was immuno-precipitated with the following antibodies: anti-p300 (#SC-585, Santa Cruz Biotechnology), anti-Ac-H4-K16 (#07-329, Millipore, Temecula, CA), anti-DNMT1 (#NB100-56519) and anti-DNMT3b (#NB300-516) from Novus Biologicals (Littleton, CO), anti-DNMT3a (#ab2850), anti-MBD2 (#ab38646), and anti-RNA Pol II (#ab5408) from Abcam and anti-rabbit IgG (#I8140) and anti-mouse IgG (#I8765) from Sigma-Aldrich. For MNase assay, nuclei were isolated from 100 mg of eAT, suspended in wash buffer (100 mmol/L Tris-HCl, 15 mmol/L NaCl, 60 mmol/L KCl, 1 mmol/L $CaCl_2$) and treated with 200 U of MNase for 20 min at 37 °C. Cross-link reversal was performed at 65 °C for at least 16 h followed by an RNase and subsequent proteinase K digestion. DNA was purified by phenol–chloroform. Samples were then run on 1% agarose gel and the resulting mononucleosomal DNA fragments (~150 bp) were gel purified. For both assays, relative protein binding and nucleosome occupancy to the *Ankrd26* gene were evaluated on recovered DNA by qPCR. Samples were normalized to their respective input using the $2^{-\Delta CT}$ method.

Electrophoretic mobility shift assay (EMSA). Protein-DNA complexes were detected using unlabeled or biotin end-labeled double-stranded DNA probes by annealing complementary oligonucleotides. Biotin 3′-end oligonucleotides, spanning the *Ankrd26* promoter sequence from −455 bp to −425 bp relative to the *Ankrd26* TSS, were from Sigma-Aldrich and, where indicated, were synthesized to incorporate methylated cytosines (^{me}C). Binding reactions, consisting of biotin-labeled probe and NE, were performed using the LightShift kit (Thermo Fisher Scientific, Waltham, MA) following manufacturer's instructions. Biotin-labeled probe (20 fmol) was added and the reaction was allowed to incubate for 20 min at room temperature. In the competition experiments, the nuclear extracts were preincubated with 200 molar excess of unlabeled probes for 20 min on ice. In super-shift experiments, 2 μg of p300 antibody (#SC-585, Santa Cruz Biotechnology) or 2 μg of rabbit IgG (#I8140, Sigma-Aldrich) was preincubated with nuclear extracts for 60 min on ice. Protein-DNA complexes were separated on native polyacrylamide gel, transferred onto nylon membrane and detected by the LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific) following manufacturer's procedure.

Primer Sequences. The list of oligonucleotides used for PCR, qPCR, MeDIP, bisulfite sequencing, ChIP, MNase, EMSA can be found as Supplementary Table S3.

Fatty Acid/BSA complex solution preparation. Palmitate and oleate have been conjugated to fatty acid-free BSA (2:1 molar ratio Fatty Acid/BSA) as described in ref. 57. Briefly, a stock solution of palmitate (100 nM) was dissolved at 70 °C in 50% ethanol in a shaking water bath. In parallel, a fatty acid-free BSA solution was prepared at 55 °C in NaCl in a shaking water bath. Finally, the palmitate and the fatty acid-free BSA solutions were complexed at 55 °C in a shacking water bath, cooled to room temperature and sterile filtered. Oleate was complexed to the fatty acid-free BSA solutions following the same protocol. For fatty acid cell treatment, control adipocytes were treated with diluent only, corresponding concentrations of BSA and ethanol.

Cell culture and transfection. 3T3-L1 cells were grown and allowed to differentiate in mature adipocytes as described[19]. Mature adipocytes were *i.* silenced with 25 nmol/l of scrambled-siRNA or *Ankrd26*-siRNA for 48 h, or *ii.* treated with palmitate (0.250 mM; Sigma-Aldrich), or oleate (0.250 mM; Sigma-Aldrich) or corresponding vehicle (diluent solution with the same concentrations of BSA and ethanol of the Fatty Acid/BSA complex solution) for 96 h, or *iii.* treated with leptin (100 nM; R&D Systems, Minneapolis, CDN) or corresponding vehicle (20 mM Tris-HCl, pH 8.0) for 24 h. Adipokines were assayed in media from silenced cells by Bio-Plex Pro Mouse Cytokine Immunoassay following the manufacturer's protocol (Bio-Rad, Hercules, CA). *Ankrd26* promoter methylation and gene expression were analyzed as previously described in this section.

Patient enrollment and tests. Abdominal VAT biopsies and serum samples were from patients undergoing bariatric surgery. Eleven normal glucose tolerance (NGT) obese subjects were selected. Population characteristics are in Supplementary Table S1. Participants with metabolic and endocrine disorders, inflammatory diseases, previous or current malignancies, and/or treated with drugs able to interfere with the epigenome were excluded. Secreted mediators were assayed in serum samples by Bioplex multiplex Human Cytokine, Chemokine and Growth factor kit (Bio-Rad) following manufacturer's protocol. *ANKRD26* gene expression was analyzed in VAT as previously described in this section.

Ethics statement. This study adhered to the Declaration of Helsinki and has been reviewed and approved by the Ethics Committee of the "Federico II" University of Naples (Ethics Approval Number: No. 225_2013). Informed consent was obtained from all of enrolled individuals.

Statistical procedures. The area under the curve (AUC) was calculated using the trapezoidal rule. Data are expressed as mean ± SD. Comparison between groups were performed using Student's t-test or the one-way analysis of variance, as appropriate, using GraphPad Software (version 6.00 for Windows, La Jolla, CA). Correlation between two variables was calculated using the parametric Pearson r-test. $p < 0.05$ was considered statistically significant.

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Acknowledgements

This study was funded by the European Foundation for the Study of Diabetes (EFSD), by the Ministero dell'Istruzione, Università e della Ricerca Scientifica (grants PRIN and FIRB-MERIT, and PON 01_02460) and by the Società Italiana di Diabetologia (SID-FO.DI.RI). This work was further supported by the P.O.R. Campania FSE 2007–2013, Project CREMe.

Author Contributions

G.A.R. conceived, designed and supervised the experiments, analysed data and wrote the paper. RS researched and analysed data and wrote the discussion. A.D., M.L., L.P., C.N., V.D.E., P.M. and F.F. collected the data. V.P. and P.FORE. contributed to the clinical study, acquisition of adipose tissue biopsies and human blood samples. P.FORM. and I.P. analysed and interpreted the data. C.M. and F.B. are the guarantors of this work and, as such, have full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. All authors critically revised and approved the final version the manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing Interests: The authors declare no competing financial interests.

How to cite this article: Raciti, G. A. *et al.* Specific CpG hyper-methylation leads to *Ankrd26* gene down-regulation in white adipose tissue of a mouse model of diet-induced obesity. *Sci. Rep.* **7**, 43526; doi: 10.1038/srep43526 (2017).

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